CHARACTERIZATION AND IDENTIFICATION OF A NOVEL GUANINE NUCLEOTIDE BINDING SITE ON THE BOVINE MITOCHONDRIAL RIBOSOME

by

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ABBREVIATIONS

Å..... Angstrom, meter x 10^{-10}

AA..... amino acid

Ac..... acetate

ACS..... American Chemical Society

ADP..... adenosine 5'-diphosphate

AR..... autoradiogram

ATP..... adenosine 5'-triphosphate

ATZ..... anilinothiazolinone

AU..... absorbance unit

$8N_3GTP$..... 8-Azido guanosine 5'-triphosphate

BCIP..... 5-bromo-4-chloro-3-indolyl phosphate

bp..... base pairs

BME..... ß-mercaptoethanol

°C..... degrees centigrade

%C..... percent crosslinker (bisacrylamide)

C4..... butyl

Ci..... curie, 2.2 x 10^{12} dpm

cm..... centimeter

cm^2..... centimeter squared

CNBR..... cyanogen bromide

cpm..... counts per minute

CTP..... cytosine 5'-triphosphate

cGMP..... 3'-5'-cyclic guanosine monophosphate

cytoribosome.. cytoplasmic ribosome

d..... day

Da..... daltons

DEAE..... diethylaminoethyl

DEPC..... diethylpyrocarbonate

dGTP..... 2'-deoxyguanosine triphosphate

DPTU..... N,N'-diphenylthiourea

DTT..... dithiothreitol

E. coli..... *Escherichia coli*

EDTA..... ethylenediaminetetraacetic acid

eIF..... eucaryotic initiation factor

EF-Tu..... bacterial elongation factor-Tu

EF-Ts..... bacterial elongation factor-Ts

EF-G..... bacterial elongation factor-G

EF-1..... eucaryotic elongation factor-1

f..... fraction

Fc..... constant fragment of IgG molecule

g..... force of gravity

GDP..... guanosine 5'-diphosphate

gm..... grams

GMP..... guanosine 5'-monophosphate

GMPPCP..... guanosine 5'-[ß,γ-methylene]triphosphate

GMPPNP..... guanosine 5'-[ß,γ-imido]triphosphate

GTP..... guanosine 5'-triphosphate

h..... hour

^{3}H..... tritium

HPLC..... high pressure liquid chromatography

IF-1..... bacterial initiation factor-1

IF-2..... bacterial initiation factor-2

IF-3..... bacterial initiation factor-3

IgG..... immunoglobulin G

Imm..... immune

ITP..... inosine 5'-triphosphate

Kd..... binding dissociation constant

kDa..... kilodaltons

Ki..... binding inhibition constant

m..... milli

M..... molar, moles per liter

mA..... milliamphere

Mr..... molecular mass

μ..... micro

μg..... microgram

μl..... microliter

μM..... micromolar

μW..... microwatt

mg..... milligram

min..... minute

mitoribosome.. mitochondrial ribosome

ml..... milliliter

mM..... millimolar

mmol..... millimole

mRNA..... messenger ribonucleic acid

mt..... mitochondria

MWM..... molecular weight markers

n..... number

NADP..... nicotinamide adenine dinucleotide phosphate

ng..... nanogram, grams x 10^{-9}

nm..... nanometer, meter x 10^{-9}

nmol..... nanomole, moles x 10^{-9}

1D..... one-dimensional

NBT..... nitro blue tetrazolium

NC..... no competition

NEM..... n-ethylmaleimide

ND..... not detected

NTP..... nucleotide triphosphate

Org..... organic

P..... peak

^{32}P..... ^{32}phosphate

PAGE..... polyacrylamide gel electrophoresis

pg..... picograms, grams x 10^{-12}

Pi..... phosphate

PI..... preimmune

PITC..... phenylisothiocyanate

pmol..... picomole, moles x 10^{-12}

POPOP..... 1,4-Bis(5-phenyloxazol-2-yl)benzene

PPO..... 2,5-diphenyloxazole

Prep..... preparation

PTH..... phenylthiohydantoin

PVDF..... poly(vinylidene difluoride) membrane

Rib..... ribosome

Res..... residue

RF..... release factor

RP-HPLC..... reverse phase-HPLC

RNA..... ribonucleic acid

rRNA..... ribosomal ribonucleic acid

S..... stained gels (used in figures)

S..... Svedberg

S5..... small subunit ribosomal protein #5 (or 1 to 33)

SD..... standard deviation

sec..... seconds

SE..... standard error

SDS..... sodium dodecyl sulfate

SW..... sperm whale

%T..... percent total monomer

TBS..... tris buffered saline

TBSA..... tris buffered saline with sodium azide

TEA..... triethanolamine

TEMED..... N,N,N',N'-Tetramethylethylenediamine

TFA..... trifluoroacetic acid

TMA..... trimethylamine

TP..... total protein

tRNA..... transfer ribonucleic acid

Tris-HCL..... tris-hydroxymethylaminomethane-hydrochloride

Triton X-100.. Octylphenoxypolyethoxyethanol

2D..... two-dimensional

UTP..... uridine 5'-triphosphate

uv..... ultraviolet

V..... volt

X..... any amino acid

Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

CHARACTERIZATION AND IDENTIFICATION OF A NOVEL GUANINE NUCLEOTIDE BINDING SITE ON THE BOVINE MITOCHONDRIAL RIBOSOME

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Bovine mitochondrial ribosomes possess a single, high affinity binding site for GTP and GDP on the small subunit. This is in contrast to procaryotic and eucaryotic cytoplasmic ribosomes which do not bind GTP directly but do so to soluble protein factors which cycle on and off the ribosome. We used a photoaffinity analog of GTP, 8-Azido GTP and well as GTP to identify ribosome components associated with the GTP binding site. 8-Azido GTP binding to the small subunit was saturable, with an apparent dissociation constant (Kd) of 1.92 μM, approximately 126 times higher than that for GTP. Photolysis of both the photoreactive analog and GTP resulted in the specific labeling of a single ribosomal protein, S5. Photolabeling of S5 by both [α^{32}P]GTP, and its 8-azido analog, was blocked competitively by the addition of excess GTP,

demonstrating the labeling was site specific. Partial amino acid sequence and analysis of this protein revealed two areas of amino acid sequence having significant homology to conserved sequences involved in GTP binding in other GTP-binding proteins. This protein with a guanine nucleotide binding site may participate in initiation complex formation on mammalian mitochondrial ribosomes. These mitochondrial ribosomes apparently employ a different mechanism for initiation of protein synthesis than bacterial or eucaryotic cytoplasmic ribosomes which are unable to interact directly with GTP.

CHAPTER 1

INTRODUCTION

An Overview of GTP-Binding Proteins

G-proteins are a central component of the cellular machinery driving olfaction, vision, control of cell proliferation, cellular regulation, and protein synthesis (for reviews see Stryer, 1986; Gilman, 1987; and Allende, 1988). Their basic function in the first four instances is to mediate signal transduction between the cell surface and the cytoplasm. This process begins when a ligand binds to the extracellular portion of a receptor protein. Upon ligand binding, a conformational change in the receptor allows binding of a GDP binding protein to the cytoplasmic portion of the same receptor. This results in the exchange of GDP for GTP on the membrane bound G-protein. This exchange of guanine nucleotides is often mediated by protein exchange factors which are an important site for regulation of these processes. This exchange results in conformational changes in the G-protein which results in dissociation of two subunits of the G-protein heterodimer, commonly called $G_{\beta,\gamma}$, leaving an activated G_{α}-GTP protein. Activated G_{α}-GTP now binds another membrane bound acceptor protein such as cGMP phosphodiesterase, adenylate

cyclase, or phospholipase C, which results in catalytic production of a second messenger molecule cGMP, cAMP, or inositol triphosphate and diacylglycerol, respectively. These soluble, second messenger molecules complete the signalling event by binding to tissue specific intracellular receptors. In the case of second messengers cGMP and cAMP, they modulate conductance of various ions through the plasma membrane. Inositol triphosphate and diacylglycerol bind to the endoplasmic reticulum, resulting in release of intracellular calcium, and to protein kinase C (PKC), forming an active PKC, respectively.

Another feature of GTP-binding proteins is an intrinsic GTPase activity which results in hydrolysis of GTP to GDP. Following activation of the acceptor molecule, the GTP-binding protein turns itself off by hydrolyzing the bound GTP to GDP resulting in an altered conformation for the protein. This results in a reduced affinity and dissociation from the acceptor protein thereby inactivating the signaling event.

In protein synthesis, GTP-binding proteins function in a different manner. There is no signaling across a cell membrane upon ligand binding and no membrane bound G-protein is involved, as described above. However, the G-proteins in this system (initiation and elongation factors) do bind GTP. Binding of GTP results in a conformational change in the factor that allows it to bind to the ribosome and produce a competent initiation or elongation complex leading to protein

synthesis. Hydrolysis of GTP, bound to these factors, provides the energy to drive both initiation and elongation in protein synthesis.

Protein Synthesis

G-Proteins are a key component in protein synthesis in both procaryotic and eucaryotic systems. The components of protein synthesis are analogous in both systems, differing primarily in the increased complexity and numbers of accessary proteins in the eucaryotic system. These accessary proteins are involved in regulation of translation in the eucaryotic system (for review see Moldave, 1985). Many features of procaryotic ribosomes resemble that of eucaryotic ribosomes therefore description of protein synthesis, will be largely based upon the accumulated knowledge of translation in procaryotic systems.

Translation of information encoded in messenger RNA (mRNA) begins with the formation of a competent ribosomal initiation complex. In E. coli, a GTP-binding initiation factor, IF-2, binds GTP and initiator fmet-tRNA on the small subunit of the ribosome forming a GTP-ternary complex. The order of binding is random and IF-2 does not necessarily bind initiator methionyl t-RNA prior to binding the ribosome (Gualaerzi, 1977, and 1986). In conjunction with two other protein initiation factors, IF-1 and IF-3, mRNA binds to complete the initiation complex. Upon formation of the complete initiation complex on the small subunit, the

intrinsic GTPase activity of IF-2 is activated and bound GTP is hydrolyzed resulting in the release of GDP-bound IF-2, IF-1, and IF-3 from the small subunit. The large subunit now binds to the small subunit with the initiator tRNA held in proper orientation with respect to the initiator codon (usually AUG) of the mRNA to form a monosome to start polypeptide elongation. IF-1 and IF-3 (which are not GTP-binding proteins) are known to increase the binding affinity of the GTP-ternary complex (Wintermeyer and Gualerzi, 1983) and IF-3 also inhibits formation of monosomes (Noll and Noll, 1972).

Eucaryotic initiation factor, eIF-2, functions in a manner nearly identical to IF-2. The major functional difference is how GDP on the inactive initiation factor exchange for GTP. The exchange of GDP for GTP occurs spontaneously in procaryotes due to relatively low binding affinities for both nucleotides. In eucaryotes the binding affinity for GDP is typically several fold greater than for GTP. An example of this is seen in Xenopus leavis oocytes (Carvallo and Allende,1987) where GDP binds 50 times better than GTP (Kd= 70 and 3800 nM for GDP and GTP, respectively). The exchange of tightly bound GDP for GTP, on EIF-2, requires a guanine exchange factor (GEF, Panniers and Henshaw, 1983) which regulates initiation of protein synthesis in eucaryotes (Ochoa, 1983).

Three elongation factors are required for protein elongation, EF-Tu/Ts, EF-G in procaryotes and EF-1/1B, EF-2 in eucaryotes. Two of these are GTP-binding proteins, EF-Tu, and EF-G (EF-1, and EF-2 in eucaryotes) while EF-Ts (EF-1B in eucaryotes) is an exchange factor. Both procaryotic and eucaryotic elongation factors function in the same manner. When GTP is bound to EF-Tu (or EF-1) a conformational change occurs allowing binding of aminoacyl-tRNA to the factor (Crane and Miller, 1974). Upon binding to the monosome and positioning of the aminoacyl-tRNA in the P-site, the intrinsic GTPase activity induces hydrolysis of GTP, and the GDP-bound EF-Tu leaves the ribosome. The exchange factor, EF-Ts, catalyses the exchange of GDP for GTP to reactivate the EF-Tu. Elongation factor EF-Tu, bound with GTP can now bind the ribosome. This induces the hydrolysis of GTP which provides the energy to transfer of the aminoacyl-tRNA to the A site. Once in the A site and the codon-anticodon pairing is correct, the peptide chain is transferred from the P site (peptidyl transferase reaction) to the amino group of the newly arrived amino-acyl-tRNA. This generates a peptidyl-tRNA. Another GTP-binding protein, EF-G catalyzes the translocation of the peptidyl-tRNA from A to the P-site. The energy to drive translocation is provided by the hydrolysis of GTP bound to EF-G.

Termination of protein synthesis in procaryotes is not as well worked out as are the initiation or elongation phases.

There are three release factors, RF-1, RF-2, and RF-3. RF-1 and RF-2 bind termination codons, UAA or UAG, and UGA, respectively in mRNA (Tate and Caskey, 1974). The binding of RF-1 or RF-2 to the ribosome stimulates the binding of the third release factor, RF-3. This factor binds GTP and it promotes the dissociation of release factors from the ribosome, completing the synthesis of the nascent polypeptide. A eucaryotic RF has been described from reticulocyte preparations (Konecki et al., 1977) which also binds GTP and has a GTPase activity.

The common elements seen in all the GTP-binding initiation, elongation, and termination factors are that they incur conformational changes upon binding GTP, that allow them to mediate the binding of aminoacylated tRNAs or other protein factors to the ribosome. Once completed they hydrolyze GTP to GDP to provide the energy to release the factor or drive protein synthesis.

Conserved Amino Acid Sequences Among GTP-Binding Proteins

Proteins that bind GTP and GDP comprise a diverse group. The group includes the polypeptide initiation and elongation factors, tubulin, transducin, adenylate cyclase receptor proteins, and a family of ras proteins. The *ras* genes encode a family of closely related proteins of approximately 21 kDa and were initially identified in human lung and bladder sarcomas (Scolnic et al., 1979; Shih et al., 1980; Papageorge et al., 1982).

Halliday (1984) described four regions of homology among various G-proteins whose amino acid sequences were known at the time. The proteins were bacterial elongation factors (EF-G and EF-Tu of *E. coli*), human c-H-*ras*1 gene product (c-H-*ras*), and yeast c-*ras*^{sc} gene product (figure 1-1). Van Meurs et al. (1987) expanded this comparison to include the α-subunits of both Tα rod and Tα cone transducin and the α-subunits of GTP-binding proteins that modulate adenylate cyclase, Gi and Gs. All sequence information for these proteins were obtained from bovine sources. Recently, several other notable G-proteins have been sequenced, a bacterial initiation factor (IF_2) and even a yeast mitochondrial elongation factor ($EF\text{-}Tu_{mt}$, see figure 1-1 for references). The same four regions of conserved sequence were found in all these GTP-binding proteins from bacterial, yeast, and mammalian sources and are shown in figure 1-1.

Two additional conserved sites for the mono-ADP ribosylation by bacterial toxins were found in the α-subunits of transducin and GTP-binding proteins that modulate adenylate cyclase (for review see Ueda and Hayaishi, 1985). ADP-ribosylation is a post-translational modification that utilizes NAD as the donor of the ADP-ribose group and leads to a decreased rate of GTP hydrolysis. This impaired intrinsic GTPase activity leaves the GTP-binding protein in a permanently activated (GTP-bound) state (Cassel and Selinger, 1977).

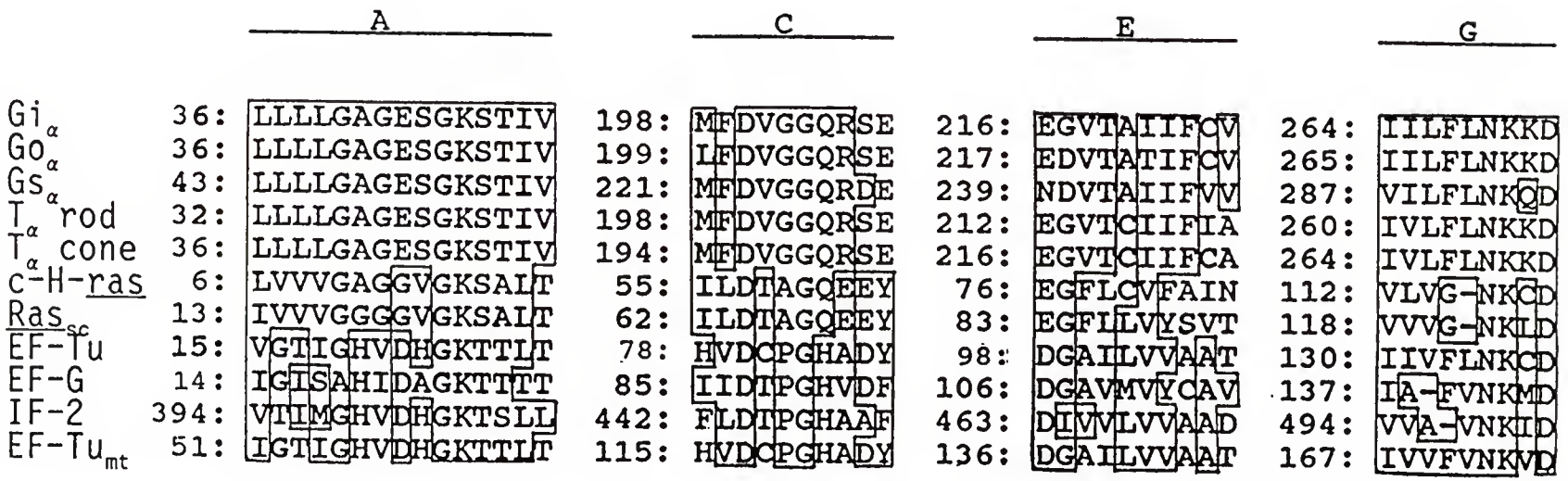

A
C
E
G
Gi$_\alpha$ 36: LLLLGAGESGKSTIV 198: MFDVGGQRSE 216: EGVTAIIFCV 264: IILFLNKKD
Go$_\alpha$ 36: LLLLGAGESGKSTIV 199: LFDVGGQRSE 217: EDVTATIFCV 265: IILFLNKKD
Gs$_\alpha$ 43: LLLLGAGESGKSTIV 221: MFDVGGQRDE 239: NDVTAIIFVV 287: VILFLNKQD
T$_\alpha$ rod 32: LLLLGAGESGKSTIV 198: MFDVGGQRSE 212: EGVTCIIFIA 260: IVLFLNKKD
T$_\alpha$ cone 36: LLLLGAGESGKSTIV 194: MFDVGGQRSE 216: EGVTCIIFCA 264: IVLFLNKKD
c-H-ras 6: LVVVGAGGVGKSALT 55: ILDTAGQEEY 76: EGFLCVFAIN 112: VLVG-NKCD
Ras$_{sc}$ 13: IVVVGGGGVGKSALT 62: ILDTAGQEEY 83: EGFILVYSVT 118: VVVG-NKLD
EF-Tu 15: VGTIGHVDHGKTTLT 78: HVDCPGHADY 98: DGAILVVAAT 130: IIVFLNKCD
EF-G 14: IGISAHIDAGKTTTT 85: IIDTPGHVDF 106: DGAVMVVCAV 137: IA-FVNKMD
IF-2 394: VTIMGHVDHGKTSLL 442: FLDTPGHAAF 463: DIVVLVVAAD 494: VVA-VNKID
EF-Tu$_{mt}$ 51: IGTIGHVDHGKTTLT 115: HVDCPGHADY 136: DGAILVVAAT 167: IVVFVNKVD

Figure 1-1 Comparison of amino acid sequences from conserved binding regions, A,C,E, and G (Halliday, 1984, and Van Meurs et al., 1987) for GTP-binding proteins that modulate adenylate cyclase Gi$_\alpha$ (inhibitory, Nukada, et al., 1986), Go$_\alpha$ (brain; Van Meurs et al., 1987), Gs$_\alpha$ (stimulatory, Robishaw et al., 1986, and Michel et al., 1986), T$_\alpha$ rod (Tanabe et al., 1985, Medynski et al., 1985, and Yatsunami and Khorana, 1985), T$_\alpha$ cone (Lochrie et al., 1985, and Lerea et al., 1986), c-H-ras (Capon et al., 1983), ras$_{sc}$ (S. cerevisiae, DeFeo-Jones et al., 1983), EF-Tu (E. coli, Jones et al., 1980, and Laursen et al., 1981), EF-G (E. coli, Ovchinnikov et al., 1982), IF-2 (E. coli, Saucerdot et al., 1984), and EF-Tu$_{mt}$ (S. cerevisiae, Nagata, et al., 1983). Boxes surround four or more residues that are identical or conserved. Residues were grouped according to Dayhoff (1978) conservative categories, C; S,T,P,A,G; N,D,E,Q; H,R,K; M,I,L,V; F,Y,W.

Cholera toxin is a ADP-ribosyltransferase that modifies the α-subunits of Gs (Cassel and Pfeuffer, 1978) and transducin (Abood et al., 1982). The ADP-ribosylation site is arginine specific, located at Arg^{174} in α-Transducin within the conserved peptide, Ser-Arg-Val-Lys.

The second enterotoxin, pertussis toxin, modifies the α-subunits of Gi(Bokoch et al., 1983 and 1984), Go (Hurley et al., 1984), and also transducin (West et al.,1985). This second ADP-ribosylation site is located within three residues of the COOH-terminus of α-transducin at Cys^{347} (S-glycoside, Hurley et al., 1984). This site resides within a conserved peptide, Asp-Cys-Gly-Leu.

Various means have been employed to identify the nucleotide binding sites of most of these proteins. The first to be well resolved was elongation factor Tu (EF-Tu) from Escherichia coli, by means of X-ray crystallographic studies (la Cour et al., 1985; Jurnak, 1985). De Vos et al. (1988) determined the crystal of cellular ras p21 to the same resolution as EF-Tu, 2.7 Å. Essential features of both structures are similar, only the structure of EF-Tu will be discussed below.

Three domains of E.coli EF-Tu were found and the best resolved was the GTP binding domain located at the NH_2 terminal portion of the protein, comprising the first 200 residues. The GTP/GDP ligand site was found to be located at the COOH terminal end of a sheet of four parallel ß strands.

The guanine nucleotide was in the anti-conformation in respect to the sugar and found in an unusual location, at the outer edge of the domain, rather than within a hydrophobic pocket. The guanine ring is partially buried in a cavity and appears to be fixed in position by the side chains of four key residues, Asn^{135}-Lys-Cys-Asp. These residues represent a highly conserved consensus sequence, of Asn-Lys-X-Asp, found in region G (figure 1-1) of all GTP-binding proteins. The ribose was exposed to the solvent. Asn^{135} and Asp^{138} lie near the guanine and can bind the keto and amino substituents of the guanine ring, respectively. Residues in both the E and G region reside in two ß-strands that interact with one side of the guanine base (Jurnak, 1985, and la Cour et al., 1985).

Mutagenesis of Lys_{136}, in conserved region G of bacterial EF-Tu to an Arg or a Glu reduces the ability of the protein to bind guanine nucleotides by about 20 or 100 fold, respectively (Hwang et al., 1989). In the ras p21 protein, mutagenesis of the corresponding Asp^{116} to Lys or Tyr in this same conserved region abolishes GTP binding activity in this protein (Shih et al., 1988).

In regions A and C (figure 1-1) are residues that interact with the pyrophosphate group. Crystallographic analysis by Jurnak (1985) and la Cour (1985) revealed that Mg^{2+} is located close to the ß-phosphate of GDP, forming a salt bridge to Asp^{80} of EF-Tu. This residue was found in the conserved peptide, Leu-Asp-Thr-Ala-Gly, in region C. In the A

region, the sequence Gly-X_4-Gly-Lys-Ser/Thr is found in all GTP-binding proteins examined so far. Lysine[24], in bacterial EF-Tu, is at the NH_2-terminus of a α-helix which has been postulated to form a positive dipole that partially neutralizes the negative charge of one the phosphates (Hol et al.,1978, Jurnak, 1985). Additionally, Ohmi et al. (1988) affinity labeled the corresponding Lys_{16} in the ras oncogene product p21 with guanosine diphospho- and triphospho-pryridoxals. These observation suggest that this residue in the conserved sequence is located in the guanine nucleotide binding site, close to the β- or γ-phosphate group of the nucleotide.

Site directed mutagenesis in a family of closely related genes, ras, which encode a group of homologous 21 Kda proteins, has yielded additional information about guanine interactions with these GTP-binding proteins. In region A the conserved region Gly^{10}-X_4-Gly-Lys-Ser/Thr in ras p21 forms a loop that interacts with the pyrophosphate group as described above. Shih et al. (1988) mutated individually the Gly residues at codons 10 and 15, to Val in ras p21 and found the GTP binding was reduced by 33 and 1000 fold, respectively.

In Harvey and Kirsten sarcoma viruses, oncogenic ras genes are found that contain a point mutation in this same conserved region, resulting in the replacement of Gly^{12} for Val (Taparowsky et al., 1982, Tabin et al., 1982, and Reddy et al., 1982). Other transforming point mutations have been

identified and are discussed fully in the review by Barbacid (1987).

Site directed mutagenesis of Gly^{12} in the cellular ras proto-oncogene resulting in the substitution of any other amino acid except proline for this residue conferred transforming properties on the ras p21 gene product. The authors postulated that α-helical structure in this region was required for transformation (Seeburg et al., 1984). Insight into the molecular events disturbed by this point mutation was brought forth by McGrath et al. (1984). They found that this single point mutation, resulting in the substitution of Gly^{12} by a Val, not only reduced GTP binding activity but also impaired the intrinsic GTPase activity of ras p21 protein by at least 10-fold. The transformation of the protoncogene product, ras p21, was attributed to this lowered rate of GTPase activity. Kinetic analysis of the hydrolysis of GTP by p21 ras revealed that the mutant (Asp^{12}) protein had a lowered rate of hydrolysis and GDP release, in comparison to that of the proto-oncogene (Val^{12}), consistent with the reduced GTPase activity found in this mutant (Neal et al., 1988).

Protein Synthesis in Mitochondria

The mechanism of protein synthesis in mammalian mitochondria is poorly understood. Mammalian mitochondrial ribosomes have been generally characterized as procaryotic in nature due to some shared antibiotic sensitivities (Denslow and O'Brien, 1974) and that both utilize f-Met-TRNA for

polypeptide initiation. However, a number of differences exist. Susceptibility to lincosamies and macrolides is much lower than in procaryotes suggesting some components of the peptidyl transferase center are altered (Denslow and O'Brien, 1978). Additionally, mammalian mitochondrial ribosomes are not sensitive to the antibiotic kirromycin, which stabilizes the binding of the EF-Tu-GTP-aminoacyl-tRNA complex to the bacterial ribosome, thereby inhibiting polypeptide elongation (Schwartzbach and Spremulli, 1989).

In regard to their fine structure and physicochemical properties, mitochondrial ribosomes differ considerably from other kinds of ribosomes. The mass of the mitoribosome is approximately the same as the procaryotic ribosome, but the buoyant density is much less. This is due to the fact that mitoribosomes contain only half the amount of rRNA and twice the protein as bacterial ribosomes (Matthews, et al., 1982). The number of ribosomal proteins is also much greater in the mitoribosome (table 1-1). In addition, mitoribosomal proteins do not appear to have any obvious homologue in the bacterial or cytoribosome as identified by electrophoretic or immunologic cross-reactivity (Pietromonaco et al., 1984 and Matthews et al., 1982). Based upon this evidence and functional studies described below, O'Brien and Matthews (1976) have assigned mitochondrial translation systems to four separate classes (Protista, fungi, plants, and animals).

Table 1-1

Protein and rRNA Content of Ribosomes

Source	Ribosome (S units)	Number of Proteins	rRNA (S units)
Bovine Liver Mitochondria	28S[a]	33	12S
	39S[b]	52	16S
	55S[c]	85	
Procaryotic E. coli	30S[a]	21	16S
	50S[b]	32	23S, 5S
	70S[c]	53	
Mammalian Eucaryotic Cytoplasmic	40S[a]	33	18S
	60S[b]	50	28S, 5.8S,5S
	80S[c]	83	

[a]Ribosome small subunit
[b]Ribosome large subunit
[c]Monosome

The mechanism for binding mRNA in mitoribosomes appears to differ from that of procaryotic and eucaryotic cytoplasmic ribosomes. In procaryotes a leader sequence 5' to the initiation codon (AUG) binds to a complementary sequence on 16S rRNA, called the Shine-Dalgarno (1974) sequence (Steitz and Jakes, 1975). This mRNA binding site is absent in mitochondrial mRNAs which often begin with the initiation codon or have very short leader sequences which have no complement on 12S rRNA (Clayton, 1984). Eucaryotic mRNAs also lack any complementary binding to rRNA. However, they do have a modified 5'-terminal modification, the $m^7G(5')ppp$ cap which functions as a recognition signal (Shatkin, 1976) for a cap

binding protein. Once the cap binding protein binds the modified mRNA it scans the 5'-untranslated mRNA leader for the proper initiation codon. Though the mechanism for initiation codon recognition in the mitoribosome is unknown, Denslow et al. (1989) did discover that the major interaction of the mRNA on the mammalian mitoribosome, covered a 30-nucleotide segment. Also, the binding of authentic mitochondrial mRNAs required homologous initiation factors.

Heterologous translocases and initiation factors from several sources were tested to determine if they would function with mammalian mitoribosomes. With few exceptions, homologous factors were required to support *in vitro* poly (U)-dependent phenylalanine polymerization in mammalian mitoribosomes. The only heterologous factor that has been shown to support poly (U)-directed systems in animal mitoribosomes was EF-Tu from a variety of sources (*E. coli*, *B. subtilis*, and a protist *Euglena gracilis*, Eberly and Spremulli, 1985). Denslow et al. (1988) also showed that bacterial IF-3 could bind to small subunits of mitoribosomes and inhibit formation of the monosome as it does in *E. coli* . Bacterial EF-G (Denslow and O'Brien, 1979) and other translocases from gram-positive and gram-negative procaryotes, protist, plants (chloroplast) and eucaryotic cytoplasmic origins did not support *in vitro* phenylalanine polymerization (Eberly et al., 1985). This was surprising in view of the fact that a postribosomal supernatant from *E. coli* would

function in a poly (U)-directed system in Neurospora crassa mitochondrial ribosomes (Grandi et al., 1971). This suggested that mammalian mitochondrial ribosomes may differ significantly from other types of mitochondrial ribosomes in their interactions with soluble initiation and elongation factors.

The first reported isolation of any elongation factors from mammalian mitochondria was EF-Tu/Ts complex from bovine liver by Schwartzbach and Spremulli (1989).

Proposal

GTP binding proteins are an essential component in protein synthetic machinery in procaryotic and eucaryotic cytoplasmic systems. Though much is known about protein synthesis in procaryotes and much more is being discovered about the eucaryotic cytoplasmic system, little is known about protein synthesis in mitochondrial systems. In a search for a putative mitochondrial elongation factor, O'Brien et al. (1990) discovered a unique GTP binding activity on the small subunit of the mammalian mitochondrial ribosome. This GTP-binding did not occur on soluble initiation or elongation factors, as is the case for other ribosomal systems, but on the surface of the mitochondrial ribosome, suggesting a novel mechanism for initiation of protein synthesis. The objective of this research was to identify the site of GTP binding on the small subunit and further characterize this binding activity. Once identified, the GTP-binding ribosomal protein was purified and partial amino acid sequence was determined in an effort to find any shared homologies to other GTP-binding proteins.

CHAPTER 2

NONCOVALENT BINDING OF MITOCHONDRIAL RIBOSOMES BY NTPs

Introduction

Guanosine-5'-triphosphate plays a key role in protein synthesis in both procaryotic and eucaryotic systems. In both systems GTP binds to soluble protein factors in a cyclic manner to promote the initiation or elongation phases of protein synthesis (Moldave, 1985, and Allende, 1988). Elucidation of the translation process in mammalian mitochondria has been difficult. The substitution of heterologous translation factors from a variety of procaryotic and eucaryotic sources generally fail to support poly (U)-directed phenylalanine synthesis with mammalian mitoribosomes. Only EF-Tu from a variety of sources was capable of functioning in mammalian mitoribosomal translation (Eberly et al., 1985) as discussed above.

In an effort to isolate a putative mitochondrial EF-G from bovine liver O'Brien et al. (1990) serendipitously found that [8-^{3}H]GTP binds to salt washed small subunits of mammalian mitochondrial ribosomes. This is surprising due to the fact that GTP-binding to activity in both procaryotic and eucaryotic cytoplasmic translation systems occurs only on

soluble protein factors that cycle on and off the ribosome in protein synthesis. This binding activity was resistant to high salt washes (300 to 500 mM KCL) and independent of any exogenous factors or fusidic which is known to stabilize EFG-GTP complex on bacterial ribosomes. Factor independent binding of GTP to procaryotic (E. coli) and eucaryotic cytoplasmic (Bovine) ribosomes has never been reported and was not observed when tested in this laboratory (Sonnega et al. personal communication).

Although no function has as yet been associated with this activity [8-^{3}H]GTP was found to bind with high affinity (Kd= 15.3 nM) and in a stoichiometric manner to salt washed 28S ribosomes (O'Brien et al., 1990). The large subunit (39S) and intact monosomes (55S) did not bind GTP. The binding site was specific for GTP and GDP and no other nucleotide tested could compete with [8-^{3}H]GTP binding to the small subunit. Binding affinity for the 28S ribosome was equally high for GDP (Kd=18 nM).

Nonhydrolyzable analogs of GTP, GMPPNP and GMPPCP, also bound to the small subunit, but with reduced affinity, suggesting that binding site is sensitive to alterations in the structure of the triphosphate side chain. Additionally, [γ^{32}P]GTP was not hydrolyzed following binding to small subunits. These findings suggest that salt washed ribosomes do not posses an intrinsic GTP hydrolytic activity. This of

course did not rule out the possibility that GTPase activity may require exogenous factors, as yet identified.

The following study will expand on the above information in an effort to determine the specificity of this GTP-binding activity. GTP-binding will be determined using standard nitrocellulose filter binding assays and under various salt conditions to determine the optimal ionic conditions for binding.

Materials and Methods

Reagents

Guanosine[8-^{3}H] 5'-Triphosphate was purchased as a ammonium salt in a 50% ethanol solution at a specific activity of 12.6 Ci/mmol and <99% purity (determined by manufacturer) from Amersham Corporation (Arlington Heights, IL). Adenosine, cytidine, guanosine, and uridine 5'[α^{32}P] Triphosphate were purchased as triethylammonium salts in aqueous solution at a specific activity of 410 Ci/mmole from Amersham Corporation. [α^{32}P] Guanosine monophosphate was purchased also as a triethylammonium salt from New England Nuclear (Boston MA) at a specific activity of approximately 3000 Ci/mmol and of at least 90% purity. 8-Azidoguanosine 5'[α^{32}P] Triphosphate, was purchased as a triethylammonium salt at a specific activity of 4.6 Ci/mmole from ICN Radiochemicals (Irvine, CA). All unlabeled nucleotide-5'-triphosphates, guanosine di- and monophosphates were Type I reagents, purchased as sodium salts from Sigma Chemical Company. The NTPs were isolated from

Equine muscle free of vanadate ion and all other nucleotides isolated from Yeast.

Mitochondrial Ribosome Preparation Solutions

Solution	Formulation
A	0.34 M sucrose, 1 mM EDTA, 5 mM Tris-HCL, pH 7.5
B	0.26 M sucrose, 40 mM KCl , 15 mM $MgCl_2$, 0.8 mM EDTA, 5 mM 2-mercaptoethanol (BME), 50 μM spermidine, 50 μM spermine, 14 mM Tris- HCl, pH 7.5
C	100 mM KCl, 20 mM $MgCl_2$, 0.01% Triton X-100, 5 mM BME, 20 mM triethanolamine (TEA), pH 7.5
D	40 mM KCl, 15 mM $MgCl_2$, 0.05 mM EDTA , 5mM BME 1.6% Triton X-100, 0.005% diethylpyrocarbonate (DEPC), 10 mM Tris-HCl, pH 7.5
E	300 mM KCl, 40 mM $MgCl_2$, 5 mM BME, 1.6% Triton X-100, 0.005% DEPC, 10mM, Tris-HCl, pH 7.5
F	300 mM KCl , 5mM $MgCl_2$, 5 mM BME, 10 mM Tris-HCl, pH 7.5
G	0.34 M sucrose, 25 mM KCl, 5 mM $MgCl_2$, 5 mM BME, 10 mM Tris-HCl, pH 7.5

Binding Assay Solutions

Reaction Buffer A: 100 mM KCl, 5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4, and 1 mM BME

Reaction Buffer B: 10 mM NH_4Cl, 20 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4, and 1 mM BME

Reaction Buffer C: 100 mM KCl, 20 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4, and 1 mM BME

ß-Scintillation Solution

Liquid Scintillation cocktail: 0.5% PPO and 0.05% (w/v) POPOP in toluene

Equipment

Beckman LS8000 ß-liquid scintillation counter

Millipore 30 place vacuum manifold

Napco Vacuum oven, model# 5831

Preparation of Mitochondrial Ribosomes

Bovine mitochondrial ribosomes were prepared from bovine liver as described previously (Matthews et al., 1982). Mitochondria prepared from liver homogenates in buffer A were treated with 100 µg/ml digitonin to remove microsomal membranes and then stored at -70°C in buffer B until needed. To isolate the ribosomes, thawed mitochondria were lysed in 1.6% Triton X-100 and clarified by centrifugation at 10,000 rpm for 45 min in a Beckman JA-10 rotor at 4°C. Bovine mitoribosomes were concentrated by absorption to DEAE cellulose in buffer D and eluted with high salt (buffer E). The crude mitoribosomes eluted from the DEAE cellulose were layered on a 34% sucrose cushion (20ml) prepared in buffer C and centrifuged at 35,000 rpm for 24 h in a Beckman type 35 rotor. The pellets were resuspended in buffer C, treated with 1 mM puromycin to remove nascent polypeptides, and centrifuged in 10-30% sucrose density gradients prepared in buffer F to yield 28S and 39S subribosomal particles. Dissociation of subribosomal particles in high salt buffer (buffer F with 300

mM KCl) removed any soluble initiation or elongation protein factors. Subribosomal particles were recovered from gradient fractions by centrifugation at 100,000 x g in a Beckman Ti 60 rotor for 15 h, resuspended in buffer G and stored at -70°C until needed.

Binding of GTP to 28S Ribosomes and Analysis by Millipore Filter Assay

Binding activity was determined by means of a Millipore filter assay. In this assay, ribosomal subunits with bound radiolabeled NTP were absorbed to nitrocellulose and washed to remove unbound NTP. The reaction mixture consisted of a fixed concentration of ribosomal protein (0.1 μM) with varying concentrations (0.05 to 5 μM) of radiolabeled NTP, in reaction buffer (A, B, or C). The specific activity of the radiolabeled NTP (specific activity was typically 410 Ci/mmol) was reduced by dilution with the appropriate unlabeled NTP just prior to use. The final volume was adjusted to 50 μl with reaction buffer in a 1.5 ml conical plastic eppendorf tube and incubated for 5 min on ice. The reaction was stopped by the addition of 0.5 ml of ice cold reaction buffer and immediately transferred to a wetted nitrocellulose filter. The filter was in a Millipore 30 place manifold under vacuum to remove the aqueous buffer. Another 0.5 ml volume of reaction buffer was used to rinse out the reaction tube and also passed through the nitrocellulose filter. The filters with bound protein were immediately washed with an additional

8 ml of ice cold reaction buffer also under vacuum. The total time for the wash steps was approximately 15 seconds.

The nitrocellulose filters were placed in empty 20 ml scintillation vials and dried under vacuum and at 70°C for approximately 1 h. The dried filters were solubilized in 5 ml of scintillation cocktail solution (0.5% PPO, and 0.05% w/v POPOP in toluene). The filters were counted in a ß-scintillation spectrometer (Beckman, model #LS8000) at a counting efficiency of 21% and 88% for ^{3}H and ^{32}P, respectively. To determine the extent of nonspecific binding of radiolabeled NTPs to nitrocellulose, control incubations were done in an identical manner, but in the absence of ribosomes. The amount of radiolabeled NTP that nonspecifically bound to the filter was then subtracted from the corresponding treatment. This nonspecific binding was typically < 1% of the experimental treatments. Additionally, total amounts of radiolabeled NTPs (the same concentration and specific activity of radiolabeled NTP used in the experiment) were determined for each experiment. This was done by pipetting an appropriate amount of radiolabeled NTP onto a wetted nitrocellulose filter without washing, drying, then counting.

Binding data were plotted the aid of personal computer using the Enzfitter Kinetics program by Leatherbarrow (1987). Data was expressed as pmol of radiolabeled NTP bound per pmol of ribosomes (R) versus the concentration of free NTP (C)

using an equation for ligand binding from Edsall and Wyman (1958) as shown below:

$$R = n(C) / Kd + C$$

where: C= concentration of free ligand; Kd= dissociation constant of ligand; and R= pmol ligand bound/ pmol ribosome

The binding data was linearly transformed by Scatchard analysis (Scatchard, 1949) by the following equation:

$$R/C = -1/Kd\ (R) + n/Kd$$

where: R= pmol ligand bound / pmol ribosome; Kd= binding dissociation constant; n= number of binding sites; C= concentration of free ligand

Binding inhibition studies were done by incubating 0.1 μM 28S ribosomes with increasing concentrations of (0.5 to 10 μM) unlabeled NTP for 1 min in reaction buffer for 1 min on ice under dim light. [α^{32}P]GTP is then added at a saturating concentration (0.1 μM) for 5 min under the same conditions. The samples are then washed and counted as described above for the amount of [α^{32}P]GTP that is bound to the ribosome in the presence of increasing concentrations of NTP.

Binding inhibition data were plotted as percent of control (Y) versus inhibitor concentration with the aid of personal computer using the Enzfitter Kinetics program by Leatherbarrow (1987). A modified equation for binding inhibition (Cantor and Schimmel, 1980) was used and is shown below:

$$Y= 1-[\ 1-r(I/Ki)/\ 1+r[1+I/Ki]\ ]x100$$

where: r= Kd/A=constant (Kd for GTP= 15.2 nM and A= concentration of GTP); Y=1-vi/vo (vi/vo = percent inhibition).

Results

Binding of NTPs to 28S Ribosomes

O'Brien et al. (1990) found that [8^{3}H]GTP binds with high affinity to 28S ribosomes and appears to be specific for GTP and GDP only. To further investigate the nucleotide specificity of binding for the small subunit, a number of NTPs were tested for their ability to bind to ribosomes and also their ability to compete for [α^{32}P]GTP binding to the 28S ribosome. Millipore filter binding assays (figure 2-1 and table 2-1) confirmed that [α^{32}P]GTP did bind to 28S ribosomes with high affinity (Kd of 14.3 nM) and in nearly unit stoichiometry which agrees with the result (Kd = 15.3 nM) of O'Brien et al. (1990). The only other [α^{32}P]NTP tested that was found to bind to this ribosome in a stoichiometric manner was GMP. GMP binds with approximately 800 times lower affinity to the 28S ribosome than GTP. [α^{32}P]ATP, UTP, and CTP did not bind to the 28S ribosome to any appreciable degree.

Binding inhibition studies were done to determine which nucleotides could displace [α^{32}P]GTP from the 28S ribosome. Denslow et al. (personal communication) found that of the NTPs (GDP, ATP, CTP, and UTP) tested only GDP was able to compete for [α^{32}P]GTP binding. In this experiment additional nucleotides were tested to determine the structural requirements for this GTP binding activity. In agreement with the results of O'Brien et al. (1990), GDP did compete strongly for [α^{32P}]GTP binding to the ribosome with a resulting Ki of

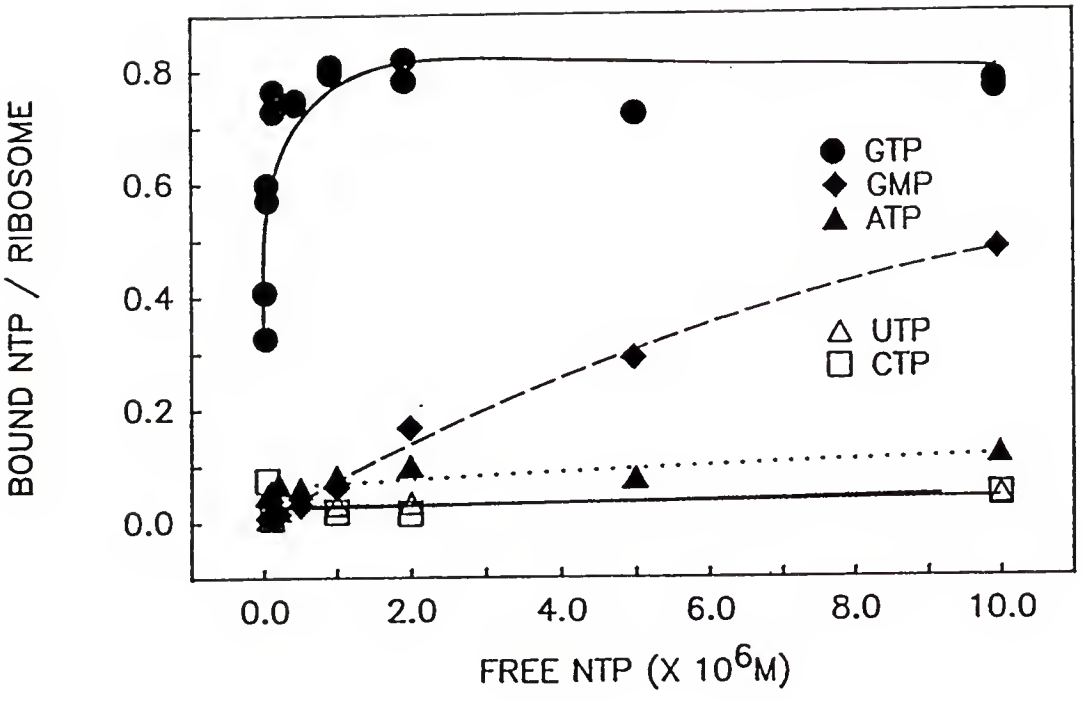


Figure 2-1 Noncovalent binding of [α^{32}P]GTP, GMP, ATP, UTP, and CTP to 28S bovine mitochondrial ribosomes. Binding of the radiolabeled nucleotides to the ribosomes was determined by the Millipore filter binding assay. The same ribosomal preparation was used for all nucleotides and the assay was done in reaction buffer A (100 mM KCL, 5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4, and 1 mM BME) as described in Methods. The initial specific activity of the [α^{32}P]NTPs was 410 Ci/mmol and was diluted approximately 160 fold with unlabeled NTP to a specific activity of 2.15, 1.67, 3.39,and 3.07 Ci/mmol for GTP, ATP, CTP, and UTP, respectively just prior to use. [α^{32}P]GMP was initially at a specific activity of 3000 Ci/mmol and diluted with GMP to a specific activity of 10.2 Ci/mmol just prior to use. The concentration of the ribosomes was 0.1 μM and the radiolabeled nucleotides varied from 0.05 to 10 μM. Each data point represents the mean of duplicate samples except for [α^{32}P]GTP where individual data points are shown without averaging. Refer to table 2-1 for Scatchard analysis of this data and to Methods for additional experimental details.

9.2 nM (figure 2-2 and table 2-2) suggesting that GTP and GDP bind to the same site on the 28S ribosome equivalently. Additionally, ITP and GMP were able to compete to a lesser extent, with binding inhibition constants (Ki) of 124 and 1140 nM, respectively.

Table 2-1

Noncovalent Binding of Various [α^{32}P] Nucleotides to 28S Ribosomes

Nucleotide	(Kd ± SE)[a] (nM)	Number Binding Sites (n ± SE)
GTP	14.1 ± 2.3	0.80 ± 0.02
GMP	11,500 ± 1,300	1.00 ± 0.06
ATP	77 ± 44	0.09 ± 0.01
CTP	ND	ND
UTP	1,200 ± 990	0.07 ± 0.02

[a]Scatchard analysis of noncovalent binding of [α^{32}P]NTP and [α^{32}P]GMP to 28S ribosomes shown in figure 2-1. Data represents binding dissociation values (Kd) and standard error (SE) from a single binding experiment. ND: Not detected

The ionic conditions used in which these binding assays were modified from those used in earlier work by O'Brien et al. (1990) in order to determine optimal stringencies for the study of this GTP binding activity. The original work by O'Brien et al. (1990) used reaction buffer B (10 mM NH_4CL, 20 mM Mg_2CL_2, 10 mM Tris-HCl, pH 7.4, and 5 mM BME) which was

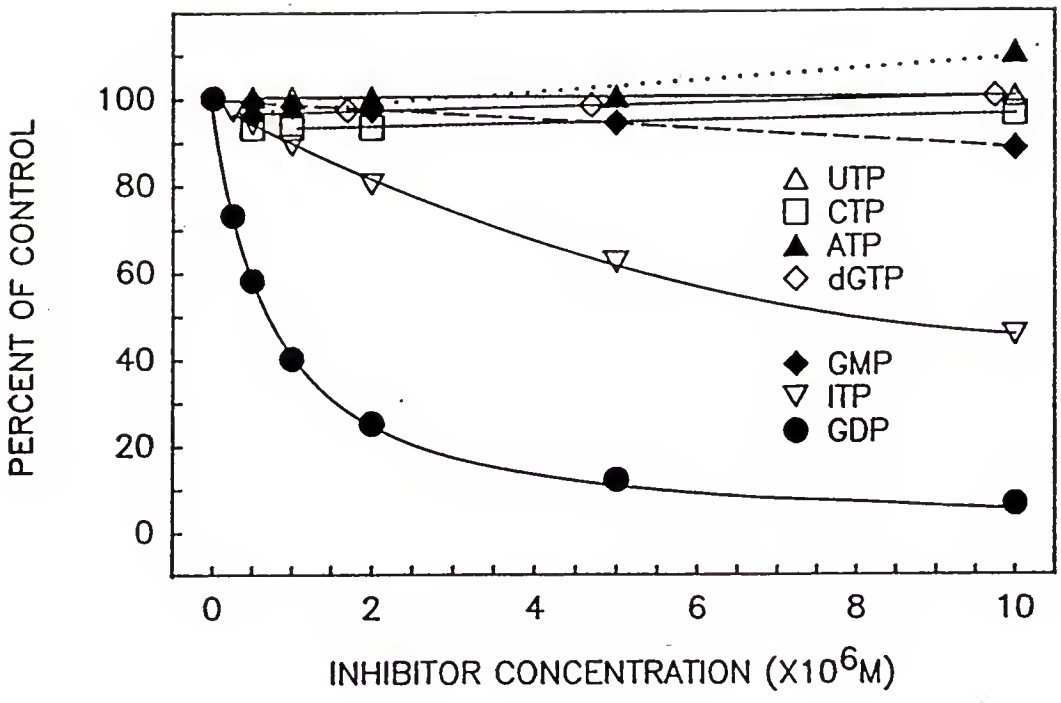


Figure 2-2 Binding inhibition of [α^{32}P]GTP to bovine mitochondrial ribosomes by various nucleotides. Displacement of bound [α^{32}P]GTP (0.1μM) from ribosomes (0.1 μM) by increasing concentrations (0.5 to 10 μM) of competing nucleotide was done in reaction buffer A as described in Methods. The [α^{32}P]GTP was diluted with GTP to a specific activity of 21.6 and 14.8 Ci/mmol just prior to use for two separate binding inhibition experiments (GDP, GMP, dGTP,ATP,CTP, and GDP, UTP, ITP respectively). The same preparation of ribosomes and chemical lot of [α^{32}P]GTP was used in both experiments. The binding of [α^{32}P]GTP to ribosomes in the presence of competing unlabeled nucleotide was compared to binding in the absence of the competing unlabeled nucleotide and represented as percent of control. The data are single data points and each curve represents a single experiment except for GDP where each data point is a mean of two experiments conducted on consecutive days. Refer to table 2-2 for analysis of binding inhibition for each nucleotide described above.

Table 2-2
Binding Inhibition for [α^{32}P] GTP Binding to 28S Ribosomes by Various Nucleotides

Nucleotide	Ki ± SE[a] (nM)
GDP	9.2 ± 1.0
ITP	124 ± 19
GMP	1,140 ± 270
dGTP	NC
CTP	NC
UTP	NC
ATP	NC

[a]Binding inhibition (Ki) of various nucleotides to compete for [α^{32}P]GTP binding to the 28S ribosome. Data represents values from a single binding experiment and standard error, shown in figure 2-2, for a single binding inhibition experiment.

NC: No binding inhibition

developed by Bodley (Bacca, et al., 1976) for the study of GTP binding to bacterial EFG. Reaction buffer A (100 mM KCl, 5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4, and 1 mM BME) was developed during the progress of the present study and was used in the above binding and inhibition binding studies. The development of these ionic conditions will be described later in this paper. At this point it suffices to say that GTP binds specifically to the 28S ribosome and quantitatively in the same manner over a wide range of ionic conditions and stringencies.

Both [8-^{3}H]GTP and [α^{32}P]8-Azido GTP Bind to the Same Site on the 28S Ribosome

In order to determine if the 8-Azido analog of GTP would be a suitable photoaffinity probe for labeling the GTP binding site on the 28S ribosome, filter binding and binding inhibition studies were performed. In an experiment directly comparing binding of [α^{32}P]8-Azido GTP and [8-^{3}H]GTP to 28S ribosomes, the GTP analog did bind to the small subunit with an apparent dissociation constant of 1.35 μM (figure 2-3). The dissociation constant for [8-^{3}H]GTP was 18.1 nM which is in close agreement with that found by O'Brien et al. (Kd=15.3 nM, 1990) and both bound in unit stoichiometry.

A mean dissociation factor for [α^{32}P]8-Azido GTP and [α^{32}P]GTP have been determined (table 2-3). These values were 1.92 ± 0.84 μM and 15.2 ± 8.64 nM, respectively. Both compounds bound to the ribosome in a stoichiometric manner (1.2 ± 0.4 and 0.84 ± 0.17 sites occupied per ribosome, respectively). These data suggest that the GTP analog binds to the 28S ribosome with an affinity approximately 125 times less than GTP. These mean Kd values for 8-Azido GTP and GTP will be used throughout the remainder of this paper.

GTP was found to compete with [α^{32}P]8-Azido GTP for binding to the 28S ribosome with an apparent Ki of 106 nM (figure 2-4). This suggests that GTP and its photoreactive analog bind to the same binding site on the 28S ribosome. These data imply that [α^{32}P]8-Azido GTP could be used as a photoaffinity probe for the identification of the GTP binding site on the 28S ribosome.

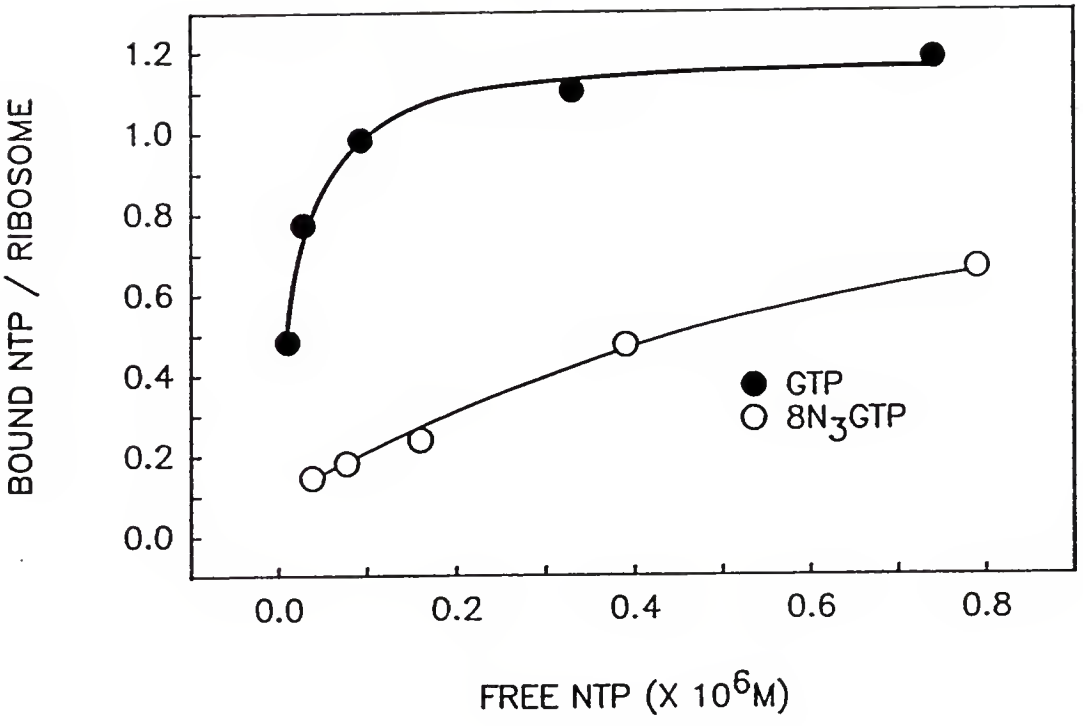


Figure 2-3 Noncovalent binding of [α^{32}P]8-Azido GTP and [8-^{3}H]GTP to 28S bovine mitochondrial ribosomes. Binding of the radiolabeled nucleotides to the ribosomes was determined by the Millipore filter binding assay as described in Methods. The same ribosomal preparation was used for both nucleotides and the assay was done in reaction buffer B (10 mM NH_4Cl, 20 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4, and 1 mM BME) as described in Methods. The specific activity of [8-^{3}H]GTP and [α^{32}P]8-Azido GTP was 3.1 and 0.98 Ci/mmol, respectively. Each data point represents the mean of duplicate samples. Scatchard analysis for the binding of GTP and its 8-Azido analog to 28S ribosomes revealed an apparent Kd of 18.1 and 1.35 μM, respectively.

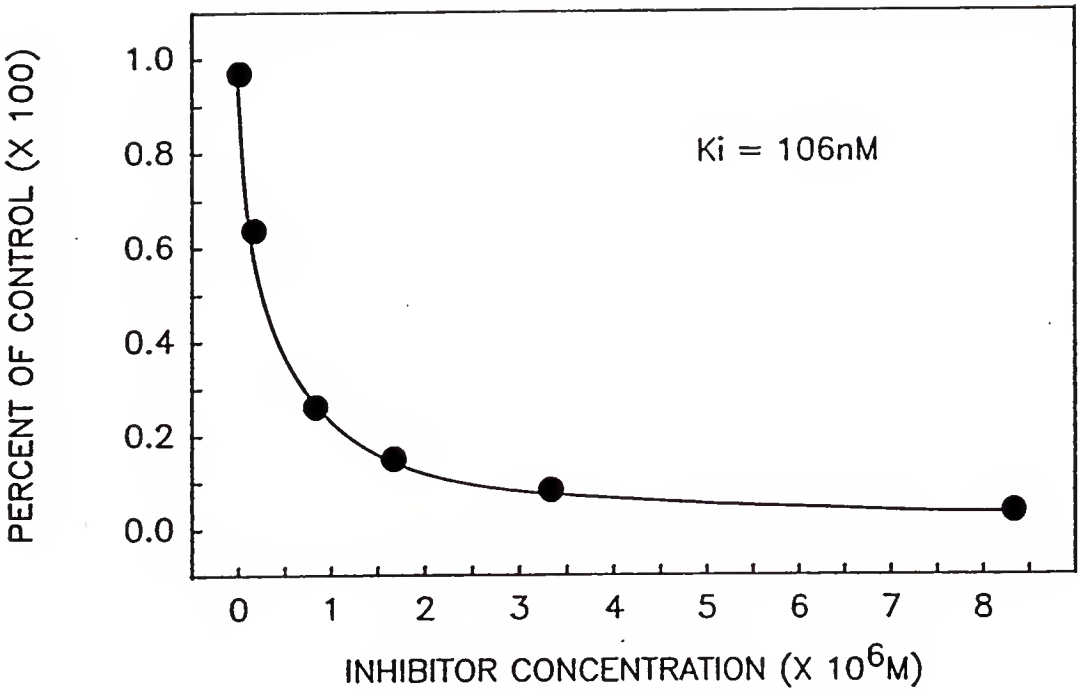


Figure 2-4 Binding inhibition of [α^{32}P]8-Azido GTP to bovine mitochondrial ribosomes by GTP. Displacement of saturating concentration (1.67 μM) of [α^{32}P]8-Azido GTP from 0.083 μM ribosomes by increasing concentrations (0 to 16.7 μM) of GTP was done in reaction buffer B as described in Methods. The specific activity of [α^{32}P]8-Azido GTP was 0.98 Ci/mmol The same preparation of ribosomes and lot of [α^{32}P]8-Azido GTP was used in this experiment and the one described in figure 2-3. The binding of [α^{32}P]8-Azido GTP to ribosomes in the presence of competing unlabeled nucleotide was compared to binding in the absence of the competing unlabeled nucleotide and represented as percent of control. The data are mean values from duplicate samples and each curve represents a single experiment. GTP was found to compete for [α^{32}P]8-Azido GTP binding to 28S ribosomes with an apparent Ki of 106 nM.

Table 2-3

Noncovalent Binding of GTP and 8-Azido GTP to 28S Ribosomes

Nucleotide	Reaction Buffer[c]	(Kd ± SD) (nM)	Binding Sites (n ± SD)
[8-^{3}H & α^{32}P]GTP[a]	A	15.2 ± 8.64	0.84 ± 0.17
[α^{32}P]8-Azido GTP[b]	B	1,920 ± 840	1.20 ± 0.40

[a]Data for the binding of [8-^{3}H] and [α^{32}P]GTP to 28S ribosomes are the mean and standard deviation of 7 experiments.

[b]Data for the binding of [α^{32}P]8-Azido GTP to 28S ribosomes are the mean and standard deviation of 3 experiments.

[c]Formulations for reaction buffers A and B are listed in Materials

Discussion

There is one (stoichiometric) nucleotide binding site on the 28S ribosome and it is specific for GTP and GDP. Additionally, O'Brien et al. (1990) suggested that the binding may occur on the interface of the ribosomes because [8-^{3}H]GTP did not bind to 55S monosomes. However it is not ruled out that monosome formation induces a conformational change in the GTP binding site, preventing binding of the nucleotide.

Only GTP and GDP bind the small subunit, and they do so equally and with high affinity. Most GTP binding proteins bind GDP more tightly than GTP, requiring an exchange factor in order to regenerate the active GTP-bound protein. However

there are several instances where GTP-binding proteins do have equal affinities for both GTP and GDP, such as the photoreceptor G-protein of frogs (Robinson et al., 1986), E. coli EF-G (Kaziro et al., 1978), and p21 ras protein (Shih et al., 1988).

The structural requirements for nucleotide binding in the GTP/GDP binding site on the 28S ribosome were elucidated from binding and binding inhibition assays as described above. No difference were seen in binding affinities for GTP vs GDP suggesting that the loss of the γ-phosphate does not perturb binding. However the loss of the ß-phosphate as seen in GMP does decrease binding affinity by at least 100-fold (table 2-2). Additionally, O'Brien et al. (1990) reported that nonhydrolyzable analogs of GTP, GMPPNP and GMPPCP, do bind the 28S ribosome, but with much reduced affinity. This was attributed to alteration of the P_{β}-X-P angles in these analogs perturbing the binding interactions with the pyrophosphate group (Yount, 1971).

Substitutions on the guanine ring also appear to be poorly tolerated (see figure 3-1 for ring numbering system). The substitution of the purine by a pyrimidine ring as seen for CTP and UTP completely abolishes the ability of the nucleotide to bind to or compete for [α^{32}P]GTP binding to the 28S ribosome (table 2-1 and 2-2, respectively). This suggest that functional groups on the base are misaligned in the single ring base, preventing interactions with the binding

site. Inosine triphosphate did compete partially with [α^{32}P]GTP binding to the small subunit suggesting that the loss of the amino group at C_2 of GTP can be tolerated to some extent. However the loss of the same amino group and the substitution of an amino for the keto group at C_6 as in ATP, abolished all binding activity, suggesting that the keto group was required for binding. Both the keto and amino group of the guanine base have been shown in crystallographic studies to bind to Asn and Asp residues, respectively in the highly conserved G region (figure 1-1) in bacterial EF-Tu and ras p21 (Jurnak, 1985, la Cour et al., 1985, and De Vos, et al., 1988).

Alterations of the ribose moiety are not tolerated either. dGTP did not displace [α^{32}P]GTP from the binding site, indicating the importance for the hydroxyl group at $C_{2'}$ on the ribose. O'Brien et al. (1990) also reported that periodate cleaved GTP did not bind the small subunit suggesting that any distortion in the conformation of the ribose affects its binding. Additionally, these investigators found that n-ethylmaleimide (NEM), a sulfhydryl modifier had no effect on GTP binding activity, suggesting that a cysteine residue is not located near critical GTP-binding residues.

The addition of the azido (N_3) moiety at C_8 of the guanine base reduces the affinity of this GTP analog for the small subunit (Anders et al., 1989). This could be attributed to stearic hindrance of the planar azido group and a change to

the syn conformation of the base preferred by 8-azido purines (Colman, 1983). In crystallographic studies of EF-Tu and ras p21 the GTP molecule resides in the GTP binding pocket in the anti conformation (Jurnak, 1985, la Cour et al., 1985, and De Vos et al., 1988) suggesting this conformational change could contribute, at least in part, to the lowered affinity of this GTP analog for the 28S ribosome.

This nucleotide binding activity on the ribosome, which is highly specific for GTP and GDP, is consistent with other GTP-binding proteins. GTP-binding proteins bind GTP in the nM to low μM concentration range and GDP commonly in the low nM range (see reviews by Stryer, 1986; Gilman, 1987; and Allende, 1988). They also have a single nucleotide binding site that is specific for GTP and GDP. In a comparison of nucleotide specificity, number of binding sites, and binding affinity, 28S ribosomes bind GTP and GDP in a similar manner as reported for other GTP-binding proteins. The only possible difference is that an intrinsic GTPase activity has not yet been observed on the salt washed 28S ribosomes. This does not rule out the possibility that an exogenous factor(s), as yet unidentified, may be required for GTP hydrolysis.

CHAPTER 3

PHOTOAFFINITY LABELING OF 28S RIBOSOMES WITH 8-AZIDO GTP

Introduction

Photoaffinity Labeling of GTP-Binding Proteins

Labeling receptor molecules with photoreactive analogs of effector molecules has become a popular method in elucidating the mechanisms by which their natural analogs regulate biological phenomena. The chemical approach to receptor site labeling is still used but suffers from the necessity to contrive a molecule that can bind in sufficient concentration to ensure preferential reaction with suitable residues that can react with the modified ligand. Most of these reagents are electrophiles which leads to selective incorporation that may not reveal the residues in close proximity to the binding site of the ligand.

Photoreactive ligands can be constructed with addition of one or just a few photosensitive constituents available today. The photoreactive moieties commonly used are either diazo- or azido-derivatives of their natural ligands (i.e.,ATP, cAMP, GTP, cGMP etc.) and which should bind their receptor, assuming sufficient latitude existed in the ligand-receptor recognition

process. They remain unreactive until irradiated with light by the investigator. Irradiation of the diazo- or azido-purine analog provides the energy to form a reactive carbene or nitrene radical, respectively, that may form a covalent bond to residues at or near the binding site in a nonselective manner. These reactive intermediates do display some electrophilic character. However, α-carbonyl carbenes and aryl nitrenes, which have been used extensively to identify nucleotide binding proteins, display wide chemical reactivity by being able to insert into C-H bonds and other heteroatomic bonds (Cooperman, 1980).

The ability to control the labeling event and provide relatively nonselective incorporation of modified ligands has made this a popular technique for the identification of purine binding proteins. With the development and commercial production of 8-Azido analogs of ATP, cAMP, GTP, and cGMP by the methods of Haley, (1976), and Czarnecki et al. (1979), the use of these reagents has increased. 8-Azido GTP was used in this study. Therefore, the following discussion will be limited to the nitrene, and not carbenes, even though the two are of the same isoelectronic specie (six valence electrons) and their chemistries are expected to be qualitatively similar. Some examples are; azido analogs of GTP have been used as photoaffinity probes to study GTP-subunit interaction in adenylate cyclase (Pfeuffer, 1977), tubulin (Potter and Haley, 1983), transducin (Kohnken and Mc Connell, 1985), and

bacterial EF-G (Girshovich and Kurtskhalia, 1979). The number of applications of these reagents is vast and the reader should consult reviews by Bayley and Knowles, 1977; and Chowdry and Westheimer, 1979).

The photoreactive azido compounds remain unreactive in the absence of light, thus allowing the investigator to bind the ligand to the binding site before activating the photoreactive reagent. Once bound, the sample is irradiated with uv light and a molecule of N_2 is photoeliminated and a reactive nitrene forms (figure 3-1). This nitrene is highly reactive (Turro, 1980; Bayley and Knowles, 1977; and Chowdhry and Westheimer, 1979) and it can insert into heteroatomic bonds of residues at or near the binding site, in a nonselective manner (Richards and Konigsberg, 1977). The type of bond formed is dependent upon the nature of the nitrene. The nitrene comes in two forms; a very reactive singlet nitrene that prefers to insert into O-H and N-H bonds, and the less reactive triplet nitrene that prefers C-H bonds (Bayley and Knowles, 1977). The nitrene atom in both species is linear in shape and the orbital occupancy is two filled$(sp^2)^4$ orbitals for the singlet, and one filled $(sp^2)^2$ and two p orbitals $(p_x)^1$ and $(p_z)^1$ for the triplet nitrene (Turro, 1980). The singlet nitrene can covalently attach itself in a single step reaction such as the addition to a C=C bond, nucleophilic attack of N-H or O-H bonds, or direct insertion into C-H bonds in the backbone or sidechains of

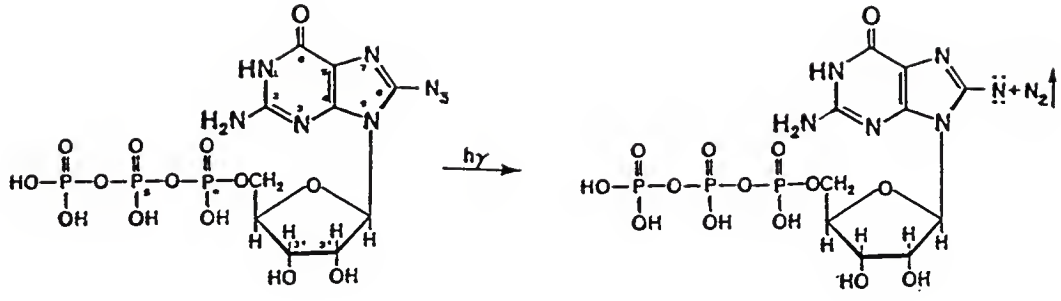


Figure 3-1 Generation of an aryl nitrene following uv irradiation of 8-Azido GTP. Formation of the reactive nitrene is accompanied by the photo-elimination of a molecule of N_2.

residues in the polypeptide residing near the nitrene while in the binding site. The triplet nitrene requires a coupled reaction before a covalent bond can form. The first reaction can be an abstraction of an electron from a C-H bond. This generates a short lived radical ($\cdot CH_3$) that can couple with reduced nitrene to form a useful covalent bond or form some other product. The only other major reactions are rearrangements, to which aryl nitrenes are not susceptible (Bayley and Knowles, 1977). The 8-Azido purine analogs do not form tetrazoles (an isomer that forms a third five membered nitrogen ring attached at C_8 to C_9 of the purine ring) in aqueous solvents to any extent (Haley, B., personal communication). These isomers of 8-Azido ATP or GTP are poor photoreactive reagents and would quench photoaffinity reactions.

The productive covalent attachment of a ligand to a receptor by photololysis is a competition between the rates of photoincorporation and rates of decomposition plus decomposition of the reagent. The rate of photoincorporation consists of the formation of the reactive nitrene which in turn must react with surrounding amino acid residues to insert into heteratomic linkages such as C=O, C-S, C-N, C-H, N-H or water. The estimates for this total reaction rate in aryl nitrenes are relatively long, approximately 10^{-4}sec when measured in a soft polystyrene matrix (Reiser et al., 1968). This suggests that if binding affinities are strong enough, at least in the millimolar range, then the ligand should reside

long enough in the binding site for productive photoinduced covalent binding to the receptor (Chowdry and Westheimer, 1979; and Potter and Haley, 1983. Additionally, the stronger the binding affinity of the ligand for its site, fewer residues will be available for interaction with the nitrene increasing the likelihood of covalently labeling a specific residue at the binding site.

Other concerns in photoaffinity labeling, besides binding affinity as discussed above, are the temperature of incubation and photolysis, ionic strength, pH, protein concentration, intensity of photolyzing light and its affect upon the receptor molecule. In the vast majority of photolabeling experiments described in the literature, the incubation and photolysis were done near 0°C and under dim red light to prevent decomposition of the azido compound. This was done in most instances to minimize the rates of exchange thereby increasing the residency of the radiolabeled ligand in the binding site and to dissipate heat from the irradiation by uv light. The ionic strength and pH should be optimal to ensure not only tight binding of ligand to the receptor but also that photoincorporation is not diminished in some manner. In the following study I will describe the photoincorporation of [α and γ^{32}]8-Azido GTP in 28S ribosomes. I will show that the GTP analog binds specifically to a single ribosomal protein and will describe the development of optimal photolabeling conditions for this reaction.

Materials and Methods

Reagents

Both [α^{32}P]8-Azido GTP and [γ^{32}P]8-Azido GTP were obtained as triethylammonium salt at specific activities of 6.7 and 10.2 Ci/mmole, respectively, from ICN radiochemicals (Irvine, CA). GTP was obtained from Sigma Chemical Co. as a Type I grade reagent.

Binding Assay Solutions

Reaction Buffer A: 100 mM KCl, 5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4, and 1 mM BME

Reaction Buffer B: 10 mM NH_4Cl, 20 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4, and 1 mM BME

Reaction Buffer C: 100 mM KCl, 20 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4, and 1 mM BME

Polyacrylamide Gel Solutions

Laemmli Sample Buffer: 2% SDS, 5% 2-BME, 10% glycerol, and 62.5 mM Tris-HCl, pH 6.8

1D-PAGE Tank Buffer: 24 mM Tris-HCl, pH 8.3, 92 mM Glycine, 0.2% SDS and 0.1 M sodium thioglycolate

2D-PAGE Sample Buffer: 9 M urea, , 10 mM 2-BME, 60 mM potassium acetate, pH 6.7, and 0.01% aminoethanethiol

2D-PAGE Prep. Buffer: 5 M urea, 2% SDS, and 10 mM sodium phosphate, pH 7.2

2D-PAGE Pre-Run Buffer: 9 M urea, 57 mM potassium hydroxide, and 340 mM HPLC grade acetic acid, pH 5.0

2D-PAGE Tank Buffer: 0.5% SDS, 0.1 M sodium phosphate, pH 7.2, 3 mM 2-BME and 0.1 mM thioglycolate.

rRNA Extraction Buffer: 15 mM sodium acetate, pH 5.0, 100 mM sodium chloride, 5 mM disodium EDTA, and 2% SDS

rRNA Run Buffer: 0.5 M Tris-HCl, pH 8.3, 0.5 M boric acid, and 10 mM EDTA.

Photolabeling of 28S Ribosomes with Radiolabeled 8-Azido-GTP

The conditions used for photoincorporation of radiolabeled 8-Azido-GTP were nearly identical to those used for the Millipore filter assay. The final reaction mixture contained 28S ribosomes (0.5 and 1.0 μM for 1D and 2D-PAGE analyses, respectively) and radiolabeled NTP at varying concentrations (0.05 to 6 μM) and GTP (5 and 10 μM for 1D and 2D-PAGE analyses, respectively) if a binding site blocker was desired. The order of addition was important. The ribosomes and GTP (if appropriate) were mixed in the reaction buffer (A, B, or C) and incubated on ice and in the dark for 5 min. The radiolabeled NTP dissolved in reaction buffer was then added to a final volume of 30 or 100 μl for 1D or 2D-PAGE analyses, respectively and incubated on ice in the dark for an additional 15 min.

Photolysis was performed with a hand held ultraviolet lamp with a purple filter that allowed transmission at 254 or 377 nm. Irradiation with 254 nm light was done in all cases and at varying distances directly above the samples resulting in intensities of 400 to 800 $\mu W/cm^2$ for photolabeling with

8-Azido-GTP or GTP, respectively. The intensity of the uv light was determined with the use of Spectroline digital uv intensity meter (model # 254X). In initial photolabeling experiments with [α^{32}P]8-Azido GTP, 2500 $\mu W/cm^2$ was used. Irradiation of samples was allowed to proceed for 5 to 10 min typically, on ice, in the dark with only dim red light for the 8-Azido GTP and with minimal room light for GTP. Samples were then prepared for either 1D or 2D PAGE analysis.

Polyacrylamide Gel Electrophoresis

Photolabeled ribosomal samples were often resolved on 1D SDS slab gels (1.5 X 160 X 200 mm) as described by Laemmli (1970). Samples were loaded into sample wells of a polyacrylamide SDS gel (12% or 15%T, 3.5%C, 0.375 M Tris-HCl, pH 8.8, and 0.1% SDS; T is the total monomer concentration, C is the crosslinker, bisacrylamide, in percentage T) with a stacker gel (3%T, 3.1%C). Electrophoresis was performed in 1D-PAGE tank buffer (24 mM Tris-HCl, pH 8.3, 0.192 M Glycine, and 0.2% SDS) at an applied constant current of 10 mA for approximately 14 h at room temperature. Following electrophoresis the gel was fixed in 50% methanol and allowed to equilibrate overnight with repeated changes of the methanol. The gel was then silver stained (Wray et al., 1981)and refixed in 50% methanol for at least 4 h to ensure complete shrinkage of the gel before autoradiography was performed. The stained and fixed gel was then sandwiched between two layers of wetted Bio-gel wrap and allowed to air

dry while clamped in a plexiglas holding frame. The dried gel was then autoradiographed at room temperature for an appropriate period of time.

A modified 2D-PAGE system of Leister and Dawid (1975) was used to determine which ribosomal protein was photolabeled by either radiolabeled 8-Azido-GTP or GTP. Following photolabeling as described above, ribosomal samples were extracted with urea and lithium chloride as described below. The first dimension was run in tube gels (3 X 110 mm) cast in siliconized (Sigmacote, Sigma) glass tubing. The acrylamide concentration of the separation tube gel was 4.6%T, 3.2%C, with 9 M urea, and 60 mM potassium acetate, pH 4.3. The first dimension was pre-run in 2D-PAGE pre-run buffer (9 M urea, 60 mM potassium acetate, pH 4.3) to remove the polymerizing agent, ammonium persulfate (0.1%). A stacker gel (4%T and 3.5%C) was then cast (3 cm long) on top of the separator tube gel. To each μl of dialyzed sample, 0.25 μl of sample gel (4%T, and 3.4%C) was added and then cast on top of the stacker gel. The first dimension was then electrophoresed at a constant amperage of 0.2 mA/gel toward the cathode in 2D-PAGE tank buffer (35 mM B-alanine, pH 4.5, and 0.01% aminoethanethiol) until the tracking dye, pyronine Y, had entered the separation gel, then the current was boosted to 0.5 mA/gel. The polymerizing agent of both stacker and sample gel was 0.1% riboflavin. When the tracker dye was within 1 cm of the end of the tube gel, electrophoresis was stopped. The

tube gel was removed from the glass tube, then equilibrated in 2D-PAGE prep. buffer (5 M urea, 2% SDS, and 10 mM sodium phosphate, pH 7.2) for 30 min while shaking gently at room temperature.

For the second dimension a SDS slab (1.5 X 160 X 200 mm) gel (10%T, and 3.5%C in 5.1 M urea, 0.5% SDS, 0.1 M sodium phosphate, pH 7.2) was used. In preformed wells adjacent to the first dimension tube gel, molecular weight markers (94, 67, 43, 30, 20.1, and 14.4 kDa) were run. The second dimension was run at a constant current of 45 mA/gel toward the anode in tank buffer (0.5%SDS, 0.1 M sodium phosphate, pH 7.2, and 3 mM BME).

The slab gels were fixed in 50% methanol, with several changes, overnight. The gels were then silver stained (Wray et al., 1981) and dried in a sandwich of Bio-gel wrap and prepared for autoradiography.

Electrophoresis of Mitochondrial 12S rRNA

Photolabeling of mitochondrial 12S rRNA, as well as ribosomal proteins , with [α^{32}P]8-Azido-GTP or [α^{32}P]GTP, was also examined. Following extraction of the photolabeled 28S ribosomes by urea and LiCl, the rRNA pellet was stored at -20°C until analyzed. The rRNA pellet was dissolved in an extraction buffer (15 mM sodium acetate, pH 5.0, 100 mM sodium chloride, 5 mM disodium EDTA, and 2% SDS) and extracted three times with an equal volume (100 µl) of equilibrated phenol followed by centrifugation at high speed in a Beckman tabletop

microcentrifuge for 5 min at room temperature. Sodium acetate was then added to a final concentration of 200 mM to the pooled aqueous phases. To precipitate the rRNA, 2.5 volumes of ice cold 95% ethanol was added and the mixture was kept at -20°C for at least 4 h. The sample was centrifuged for 10 min in a microcentrifuge at high speed to pellet the rRNA. The rRNA pellet was washed two more times with 70% ethanol followed by a final wash with 95% ethanol. The sample was dried under vacuum for approximately 15 min.

The rRNA pellet was redissolved in a 1:1 ratio of rRNA to formamide loading buffer (80% formamide, 10 mM EDTA, pH 8, 1 mg/ml bromophenol blue, and 1 mg/ml xylene cyanol FF) prior to 1D urea PAGE. Samples were resolved with molecular markers of six synthetic poly(A)-tailed RNAs ranging in length from 0.24 to 9.49 kb (BRL, Gaithersburg, MD) on a one dimensional urea PAGE (7.5%T, 3.5%C, 7 M urea, 0.5 M Tris-HCl, pH 8.3, 0.5 M boric acid, 10 mM EDTA, 0.05% TEMED, and 0.017% ammonium persulfate) with a stacker gel (5%T, 3.5%C). Electrophoresis was done in rRNA run buffer (0.5 M Tris-HCl, pH 8.3, 0.5 M boric acid, and 10 mM EDTA) at a constant voltage of 500 V toward the anode. The gel was then soaked in 0.2 M sodium acetate, pH 5.0, for 30 min prior to staining with methylene blue. The stained gel was washed with water, destained in 5% acetic acid overnight, and then washed with water again just prior to photography. The gel was dried overnight in a sandwich of Bio-gel wrap and prepared for autoradiography.

Extraction of Ribosomal Proteins for 2D-PAGE Electrophoretic Analysis

Ribosomes were extracted in urea-LiCl to remove rRNA from ribosomal proteins as described by Matthews et al. (1982) prior to 2D-PAGE analysis. Thawed 28S ribosomes were adjusted to 9 M urea, 3 M LiCl with addition of solid urea and LiCl with a resulting neutral pH. The samples were stirred overnight at 4°C followed by centrifugation at 50,000 rpm (100,000 X g) for 60 min in a Beckman type 65 rotor to precipitate the rRNA. The supernatant containing ribosomal proteins was removed and the rRNA pellet was extracted once again, by stirring for 2 h at 4°C, with the above buffer. After centrifugation the supernatants were pooled and dialyzed against 2D-PAGE sample buffer (9 M urea, 0.01% aminoethanethiol, 10 mM BME, and 60 mM potassium acetate, pH 6.7) overnight at room temperature in preparation for 2D-PAGE analysis. The rRNA pellets were stored in the type 65 centrifuge tubes at -20°C until needed.

Results

[α^{32}P]8-Azido GTP Photolabels a 38 kDa Ribosomal Protein in a Specific Manner.

Filter binding studies suggest that a single and specific binding site for GTP exists on the mitochondrial ribosomal small subunit. To identify which ribosomal protein(s) may be involved a photochemical approach was used. 28S ribosomes were incubated with increasing concentrations (0.15 to 6.0 μM) of [α^{32}P]8-Azido GTP in reaction buffer B.

The samples were irradiated with uv (254 nm) light for 20 min at 2500 $\mu W/cm^2$ on ice (and in the dark). The photoaffinity labeled ribosomes were resolved on a 12% polyacrylamide Laemmli gel, silver stained and prepared for autoradiography as described in Methods. Photoaffinity labeling of ribosomal small subunits with [α^{32}P]8-Azido GTP yielded a single specifically radiolabeled 38 kDa ribosomal protein (figure 3-2A). The addition of excess unlabeled GTP blocked photolabeling of this protein, suggesting that the binding site was also specific for GTP (figure 3-2 A). The amount of radiolabeling increased as the concentration of the [α^{32}P]8-Azido GTP increased but is approaching saturation at 6μM [α^{32}P]8-Azido GTP. This is consistent with the apparent Kd of 1.92 μM (75% of the binding sites on 0.5μM 28S ribosomes should be occupied at this Kd and concentration of 8-Azido GTP used in this experiment) determined by filter binding assays (table 2-3). Several ribosomal proteins with Mr > 40 kDa were nonspecifically photolabeled at concentrations of [α^{32}P]8-Azido GTP above the Kd for 8-Azido GTP.

Unambiguous identification of the specifically photolabeled 40 kDa ribosomal protein was not possible by one dimensional gel analysis due to the existence of other 28S ribosomal proteins of similar molecular weight. Two dimensional gel analysis of photoaffinity labeled small subunits was done in order to do so. In figure 3-3 a single ribosomal protein, S5, was specifically photolabeled with [α^{32}P]8-Azido

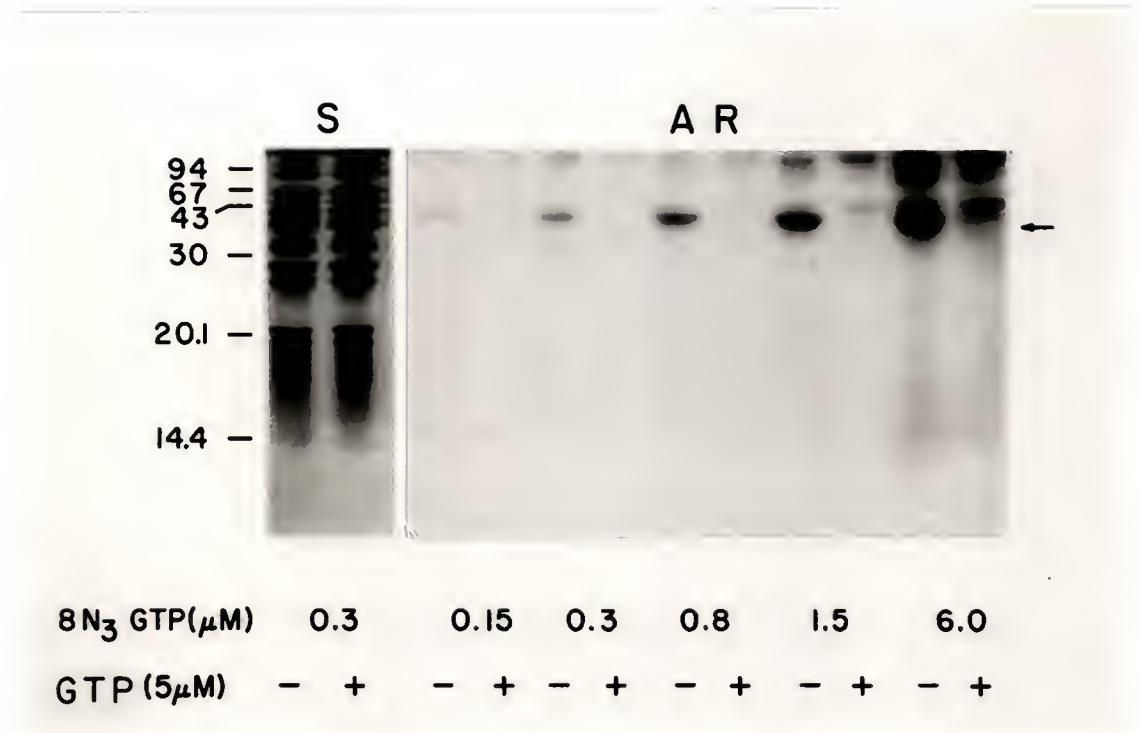


Figure 3-2 A Photoaffinity labeling of 28S ribosomes with [α^{32}P]8-Azido GTP. Bovine 28S ribosomes (0.5 μM) were irradiated in the presence of increasing concentrations (0.15 to 6 μM) of the 8-azido analog of GTP in reaction buffer B. The samples were irradiated with uv light for 20 min at 2500 μW/cm^2 on ice under dim red light. The specific activity of the [α^{32}P]8-Azido GTP was 1.35 Ci/mmol just prior to use. In panel A a representative lane of silver stained (S) proteins electrophoresed on a 12% SDS-polyacrylamide gel and the corresponding autoradiogram (AR) are shown. In alternate assays the GTP binding site was blocked by the addition (+) of 5μM GTP. The Mr markers were as follows: Phosphorylase b (94 kDa), Bovine Serum Albumin (67 kDa), Ovalbumin (43 kDa), Carbonic Anhydrase (30 kDa), Soybean Trypsin Inhibitor (20.1 kDa), and alpha-Lactalbumin (14.4 kDa). These same Mr markers were used throughout this entire study. The arrow indicates the presence of a 38 kDa protein specifically radiolabeled with [α^{32}P]8-Azido GTP. See Methods for additional details.

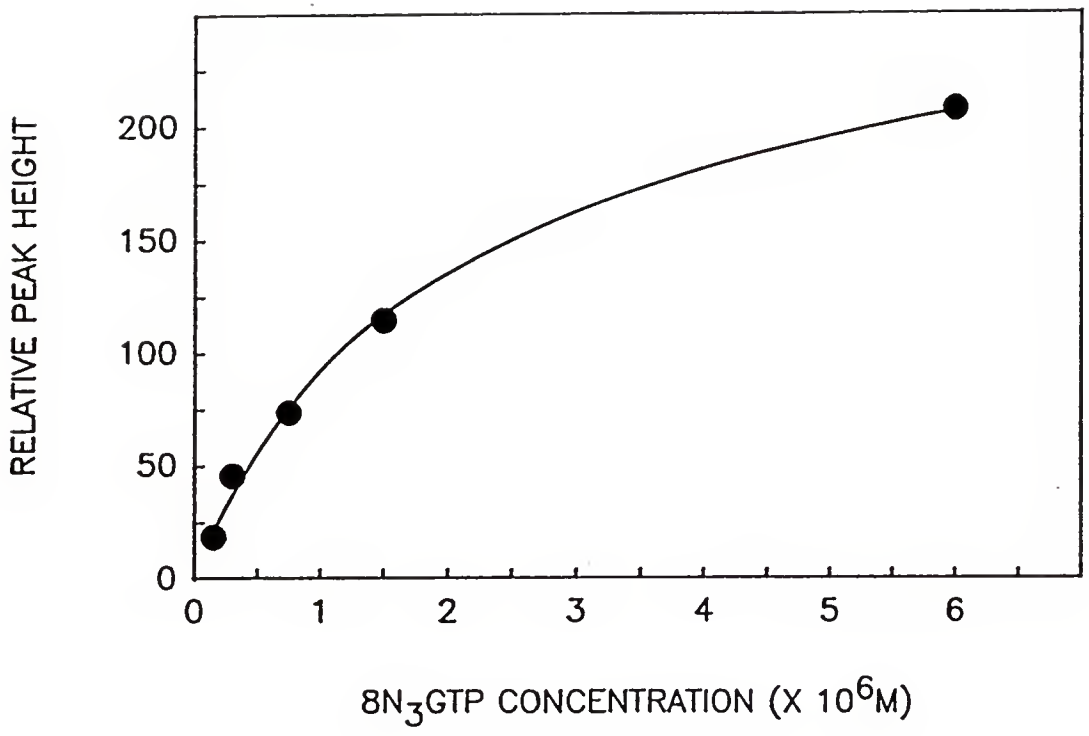


Figure 3-2 B Densitometric analysis of the autoradiogram for the specifically radiolabeled 38 kDa ribosomal protein in figure 3-2 A is shown. Data were the relative peak height densitometric measurements of the 38 kDa band (panel A) in the presence of increasing concentrations of [α^{32}P]8-Azido GTP and the absence of GTP.

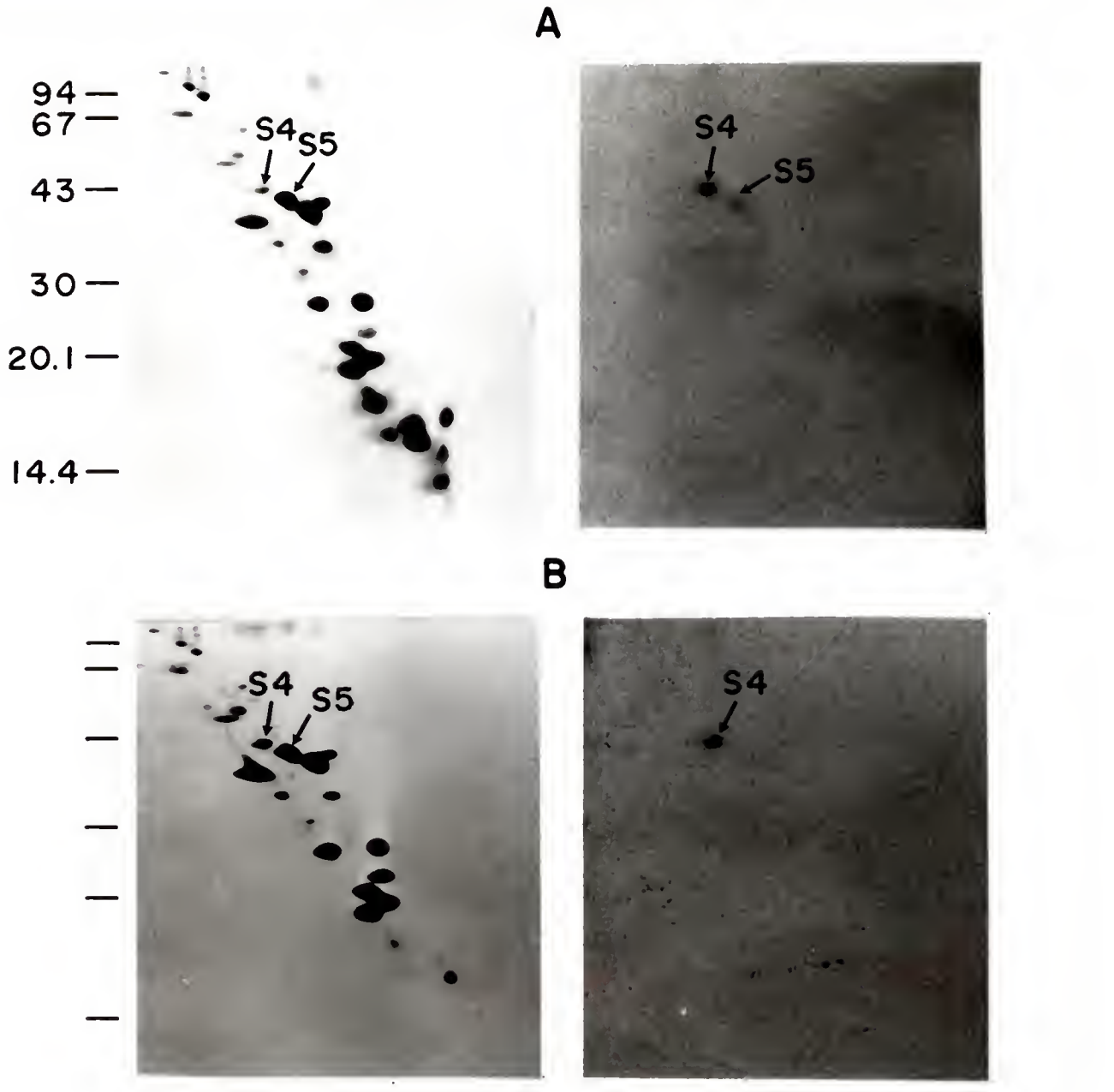


Figure 3-3 Two dimensional PAGE analysis of photoaffinity labeled 28S ribosomes with [α^{32}P]8-Azido GTP. Bovine 28S ribosomes (1 μM) were irradiated in the presence of 2 μM [α^{32}P]8-Azido GTP (45% saturation) and in the presence (B) and absence (A) of 10 μM GTP. The samples were irradiated with uv light for 10 min at 2500 μW/cm^2 on ice as described in Methods. The specific activity of the [α^{32}P]8-Azido GTP was 1 Ci/mmol just prior to use. Silver stained 2D-PAGE gels are on the left and their corresponding autoradiograms are on the right. Autoradiograms were exposed for 14 days. The molecular weight markers used were the same ones described in figure 3-2. The arrows indicate the presence of the nonspecifically labeled (S4) and the specifically labeled (S5) proteins. See Methods for additional experimental details.

GTP. The presence of excess amounts of unlabeled GTP completely blocked photoincorporation of [α^{32}P]8-Azido GTP into S5. Another ribosomal protein, S4, was also labeled nonspecifically, since the presence of excess amounts of GTP did not block photolabeling of S4.

Development of Optimal Photoaffinity Labeling Conditions for 8-Azido GTP

The extent of specific photolabeling of S5 by [α^{32}P]8-Azido GTP was small, especially in comparison to the much greater nonspecific labeling of S4 (figure 3-3). Optimization of photoirradiation and ionic conditions was undertaken to improve the quality of the photoaffinity labeling of 28S ribosomes by 8-Azido GTP. Optimal conditions were determined by both filter binding assays and photoaffinity labeling methods.

The development of optimal ionic conditions focused on the modification of two components of the reaction buffer B as developed by Bodley (Bacca, et al., 1976). Bodley developed this reaction buffer for the study of GTP hydrolysis by EFG in bacterial systems. One component, NH_4Cl, is known to be toxic to peptidyl transferase activity (Denslow and O'Brien, personal communication) on the large subunit of the mitochondrial ribosome and its replacement with another monovalent salt such as KCl was studied.

In table 3-1 the effect of replacing 10 mM NH_4Cl with KCl on the binding of [8-^{3}H]GTP to 28S ribosomes was examined by

filter binding assays. Increasing concentrations of [α^{32}P]GTP were incubated with 0.1 μM 28S ribosomes in reaction buffer B (treatment #1 in table 3-1) as a control, and the test reactions were in the same reaction buffer but with 10 mM NH_4Cl replaced with increasing concentrations (10 to 100 mM) of KCl and decreasing concentrations of $MgCl_2$. The substitution of NH_4Cl by KCl or the reduction of $MgCl_2$ from 20 to 5 mM did not alter the binding of [α^{32}P]GTP to 28S ribosomes. The most stringent condition, 100 mM KCl and 20 mM $MgCl_2$, supports binding of [8-^{3}H]GTP to the 28S ribosome in an equivalent manner as controls. This reaction buffer C (100 mM KCl, 20 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4, and 1 mM BME) and was used in experiments discussed below.

Potter and Haley (1983) reported that in their experience with a number of azido-purine analogs, photolysis at 254 nm and at intensities of 180 to 800 μW/cm^2 for 1 to 10 min caused little if any detectable damage in a variety of enzymatic systems. In our initial experiments (figure 3-2 and 3-3) these guidelines were exceeded.

The intensity and duration of photoirradiation was reduced to evaluate the minimal amount of uv irradiation necessary to adequately photolabel 28S ribosomes with [α^{32}P]8-Azido GTP. Ribosomes (0.5 μM) were photolabeled in the presence of 0.75 μM [α^{32}P]8-Azido GTP, corresponding to approximately 24% saturation of the GTP binding site (calculation based upon a Kd of 1.92 μM) in reaction buffer B.

Table 3-1

The Effect of Substituting KCl for NH_4CL and the Lowering of the $MgCl_2$ Concentration in the Reaction Buffer on the Noncovalent Binding of [α^{32}P] GTP to 28S Ribosomes

Treatment #	NH_4Cl (mM)	$MgCl_2$ (mM)	KCl (mM)	μ^a (mM)	Kd ± SE (nM)	n ± SE
1 (B)	10	20	-	75	3.8 ± 2.2	0.91 ± 0.08
2	-	20	10	75	4.0 ± 1.0	0.99 ± 0.05
3	-	20	20	85	3.1 ± 0.8	0.92 ± 0.04
4	-	20	40	105	1.7 ± 1.0	0.96 ± 0.11
5	-	20	60	125	8.3 ± 3.4	0.91 ± 0.07
6 (C)	-	20	100	165	5.8 ± 2.5	0.87 ± 0.06
7	-	10	100	135	2.5 ± 0.9	0.92 ± 0.06
8 (A)	-	5	100	120	2.7 ± 0.8	1.00 ± 0.06

Scatchard analysis for the binding of [α^{32}P]GTP to 28S ribosomes under various ionic conditions by the Millipore filter assay. The data are binding affinities (Kd) and number of binding sites (n) for a single binding curve. The concentration of the ribosomes was 0.1 μM and the [α^{32}P]GTP 0.02 to 1 μM. The specific activity of the [α^{32}P]GTP was diluted to 25.4 Ci/mmol with GTP just prior to use. Reaction buffers A, B, and C (for complete formulations see Methods) are anotated in parentheses, beside their respective treatments . In all reaction buffers 1mM BME was used. No differences were observed with the substitution of NH_4Cl in reaction buffer B (treatment #1) for increasing concentrations of KCl (treatments 2 to 6) or decreasing concentrations of $MgCl_2$ (treatments 7 & 8).

[a]μ = total ionic strength

In figure 3-4 A the reduction of 254 nm uv irradiation from 2500 to 800 μW/cm^2 for 10 min reduced the photolabeling of the 38 kDa ribosomal protein (no excess GTP present) by only 47%, as determined by densitometric analysis (figure 3-4 B). The amount of labeling was still adequate for analysis. However, irradiation for 2 min reduced photolabeling of the same 38 kDa protein by 75 to 90% at 2500 and 800 μW/cm^2, respectively.

This provided minimal detectable labeling at 2500 $\mu W/cm^2$ and virtually no labeling at the lower level of uv irradiation even after a 2 week exposure of the autoradiogram. The pattern of photolabeling remained the same as seen in figure 3-2 A. Photolabeling of the 38 kDa protein by [α^{32}P]8-Azido GTP was blocked in the presence of excess GTP and not in the case of the 43 kDa ribosomal protein.

In an continuing effort to find the minimal exposure of ribosomes with uv light for adequate photolabeling [γ^{32}P]8-Azido GTP was used. This isotope was used because [α^{32}P]8-Azido GTP was no longer available. [γ^{32}P]8-Azido GTP was also available in higher purity (>98%), more than twice the specific activity (10.2 Ci/mmole),and at lower cost. Optimal photoirradiation conditions for the photolabeling of 28S ribosomes with [γ^{32}P]8-Azido GTP were determined in reaction buffer C (100 mM KCl, 20 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4, and 1 mM BME). Substoichiometric concentrations of [γ^{32}P]8-Azido GTP (0.43 μM), corresponding to approximately 18% binding, were incubated with 28S ribosomes (0.5 μM) and irradiated at 300 and 800 $\mu W/cm^2$ for 4 and 8 min on ice in the dark. The minimal uv (254 nm) irradiation intensity and duration found for adequate labeling were 300 $\mu W/cm^2$ for 10 min (figure 3-5 A and B). These conditions were utilized in subsequent studies involving this analog of GTP.

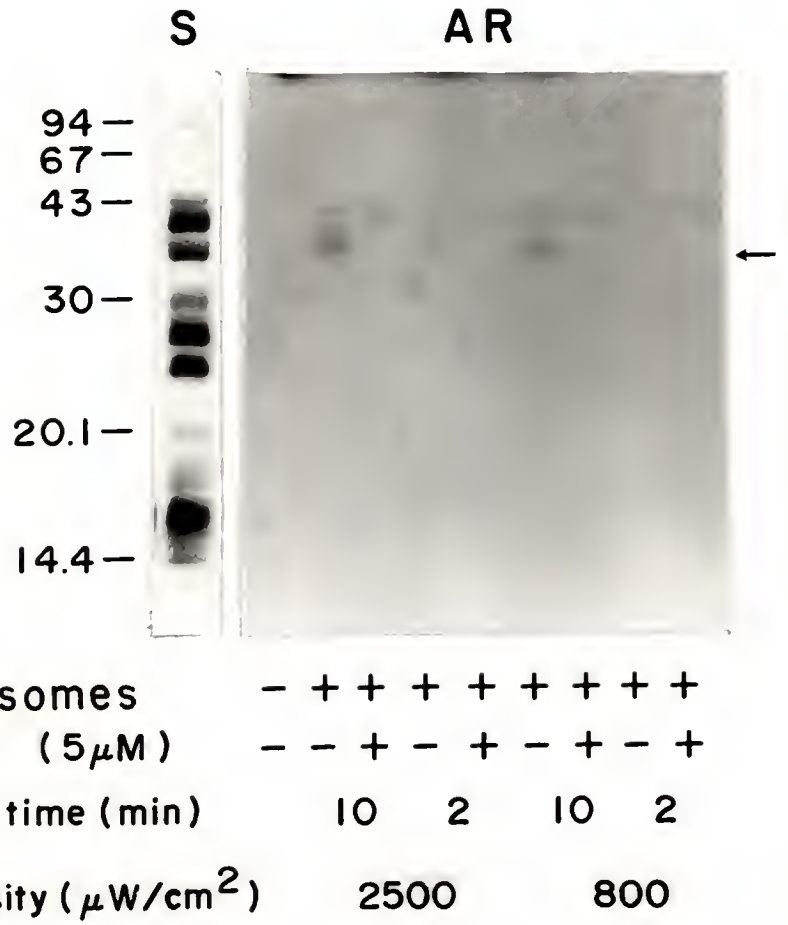


Figure 3-4 A Optimization of the intensity and duration of uv irradiation for photoaffinity labeling of 28S ribosomes with [α^{32}P]8-Azido GTP. 28S ribosomes (0.5 μM) were incubated with 0.75 μM [α^{32}P]8-Azido GTP (24% saturation) in reaction buffer B. The specific activity of the [α^{32}P]8-Azido GTP was 1.0 Ci/mmol. In panel A is a representative lane of silver stained ribosomal proteins on the left and the corresponding autoradiogram on the right. The AR was exposed for 14 days before development. The presence (+) and absence (-) of ribosomes and unlabeled GTP as well as the intensity and duration of uv (254 nm) irradiation are noted below the autoradiogram. The arrow indicates the position of the specifically radiolabeled 38 kDa protein. For more experimental details see Methods.

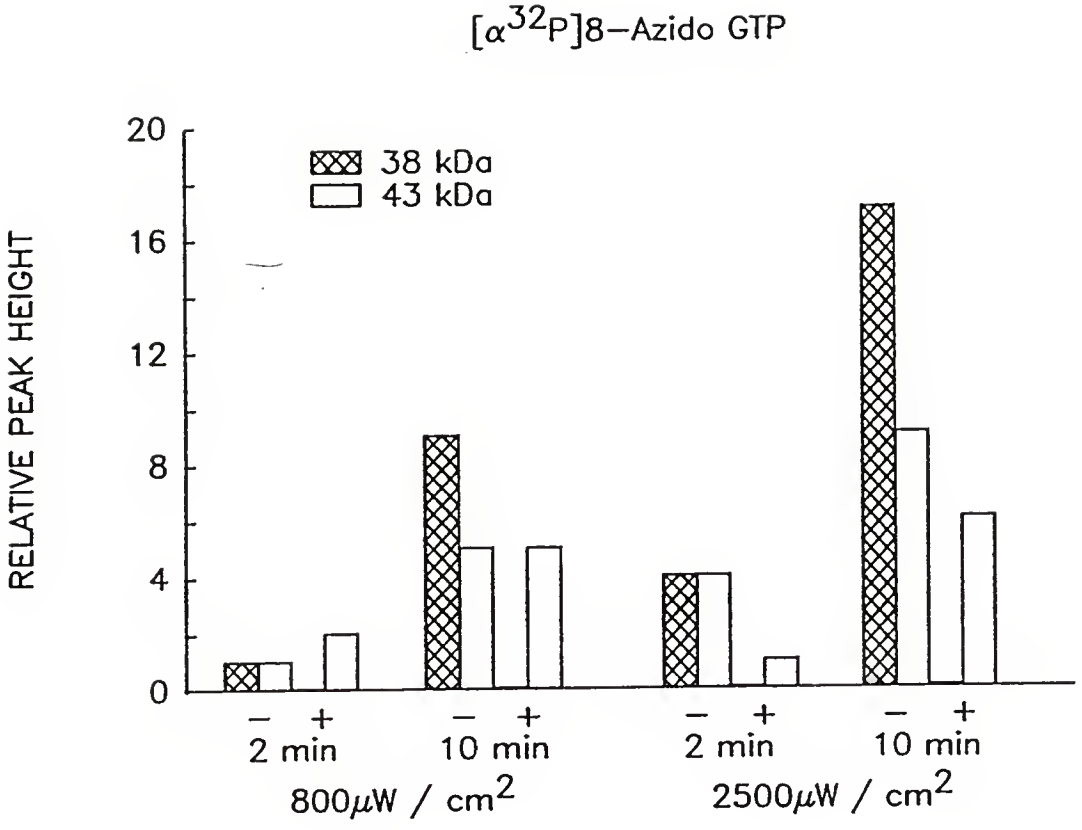


Figure 3-4 B Densitometric analysis of the autoradiogram in figure 3-4 A is shown. Densitometric analysis was done for the specifically radiolabeled ribosomal protein (38 kDa) and the nonspecifically labeled protein (43 kDa) ribosomal protein at varying intensities and duration of uv irradiation. Note that in the presence (+) of 5 μM GTP the 38 kDa protein was not radiolabeled. For additional experimental details see Methods.

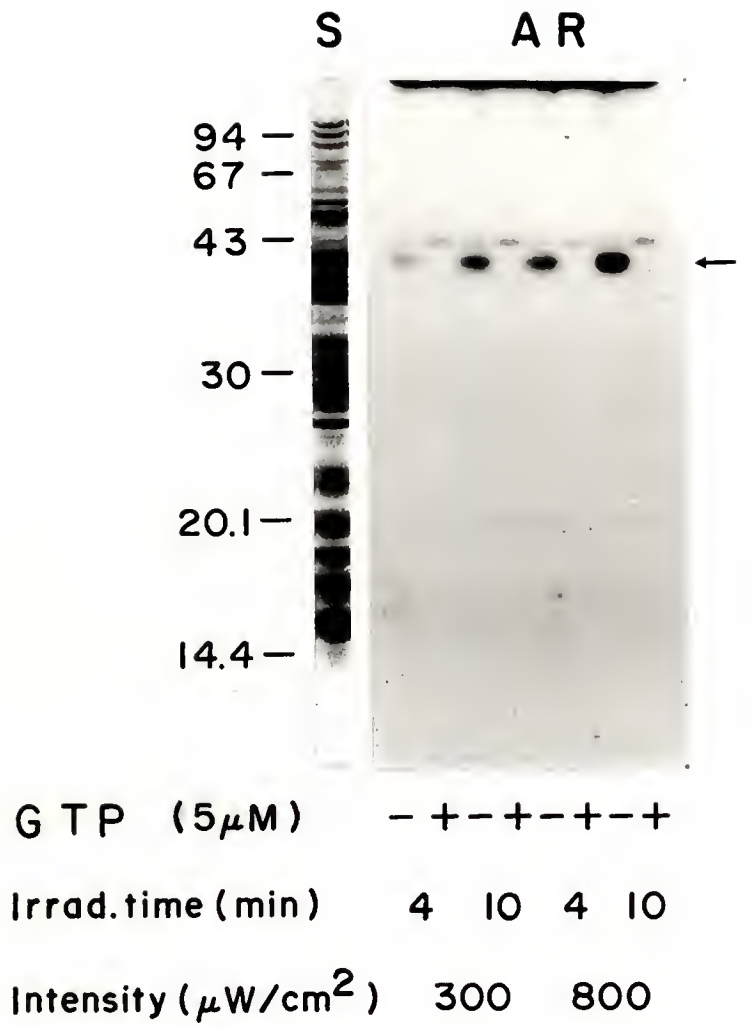


Figure 3-5 A Continued optimization of the intensity and duration of uv irradiation for photoaffinity labeling of 28S ribosomes with [γ^{32}P]8-Azido GTP. 28S ribosomes (0.5 μM) were incubated with 0.5 μM [γ^{32}P]8-Azido GTP (18% saturation) in reaction buffer C. The specific activity of the [γ^{32}P]8-Azido GTP was 8.4 Ci/mmol. In panel A is a representative lane of silver stained (S) ribosomal proteins on the left and the corresponding autoradiogram (AR) on the right. The AR was exposed for 3 days before development. The presence (+) and absence (-) of unlabeled GTP as well as the intensity and duration of uv (254 nm) irradiation are noted below the autoradiogram.

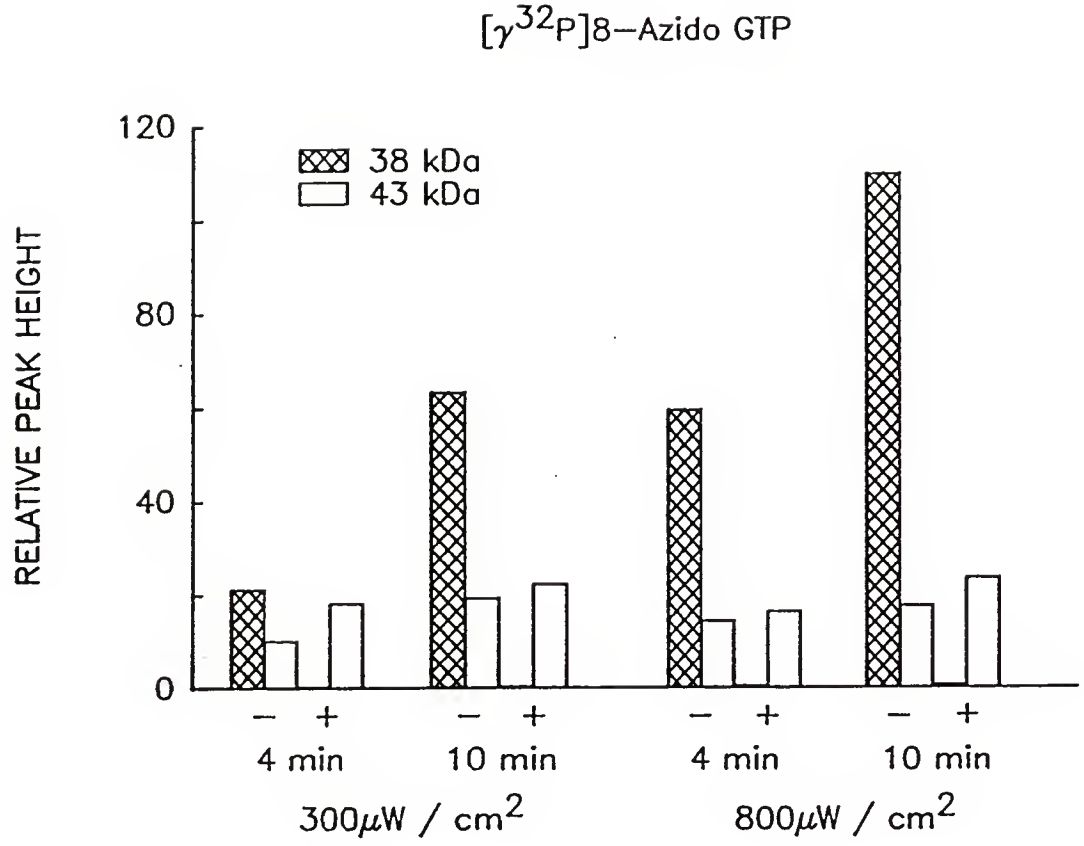


Figure 3-5 B Densitometric analysis of the autoradiogram in figure 3-5 A is shown. Densitometric analysis was done for the specifically labeled ribosomal protein (38 kDa) and the nonspecifically labeled protein (43 kDa) ribosomal protein at varying intensities and duration of uv irradiation. The presence (+) and absence (-) of 5 μM GTP is indicated below the figure. In the presence of excess amounts of GTP (+) the 38 kDa protein was not radiolabeled. For additional experimental details see Methods.

No significant differences were seen in the photolabeling pattern of the two isotopes (compare figures 3-4 A and 3-5 A) though the intensity of radiolabeling was greater for the [γ^{32}P] isotope due to its higher specific activity. The [γ^{32}P]8-Azido GTP was utilized in the remainder of 8-Azido GTP photoaffinity experiments described in this paper.

The last component of the reaction buffer to be modified was the divalent cation, $MgCl_2$. $MgCl_2$ has been shown by Denslow et al. (1988) to promote adherence of 28 and 39S subunits to one another due presumably to salt bridging effects at higher concentrations of the divalent cation. To reduce the possibility of nonspecific binding due to salt bridging of the nucleotide, $MgCl_2$ concentration was reduced from 20 to 5 mM and photolabeling was examined under these conditions. The same incubation conditions were used as described in the above experiment and uv photoirradiation was done at 300 $\mu W/cm^2$ for 10 min. The 38 kDa ribosomal protein was still specifically photolabeled (figure 3-6 A) by the GTP analog, [γ^{32}P]8-Azido GTP, and a 45% increase in this labeling was observed by densitometric analysis as the concentration of $MgCl_2$ dropped (figure 3-6 B) to 5 mM. Since the lowest concentration of $MgCl_2$ provided a modest increase in photolabeling of the specifically labeled 38 kDa ribosomal protein it was adopted for subsequent studies. The amount oflabeling on the nonspecifically labeled 43 kDa ribosomal protein was unchanged in the absence of excess GTP but did increase 47% in the presence of 5 mM GTP.

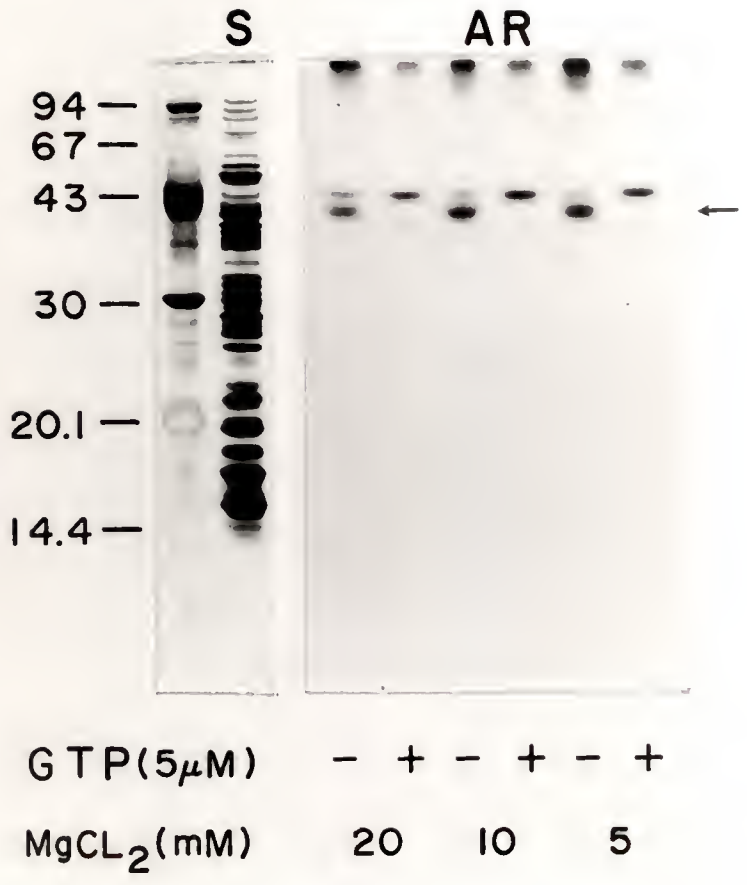


Figure 3-6 A The effect of decreasing concentrations of $MgCl_2$ on photoaffinity labeling of 28S ribosomes with [γ^{32}P]8-Azido GTP. Bovine 28S ribosomes (0.5 μM) were incubated with 0.5 μM [γ^{32}P]8-Azido GTP (18% saturation) and irradiated with uv light for 10 min at 300 μW/cm^2 on ice. The specific activity of the [γ^{32}P]8-Azido GTP was 6.3 Ci/mmol. The concentration of the $MgCl_2$ component of the reaction buffer was decreased from 20 mM (reaction buffer C) to 5 mM (reaction buffer A). In panel A a representative silver stained lane of 28S ribosomal proteins and molecular weight markers are shown with the corresponding autoradiogram on the right. The concentration of the $MgCl_2$ component of the reaction buffer and the presence (+) and absence (-) of 5 μM unlabeled GTP is shown below the autoradiogram. The autoradiogram was exposed for 3.5 days before developing. The arrow indicates the position of the 38 kDa specifically radiolabeled protein.

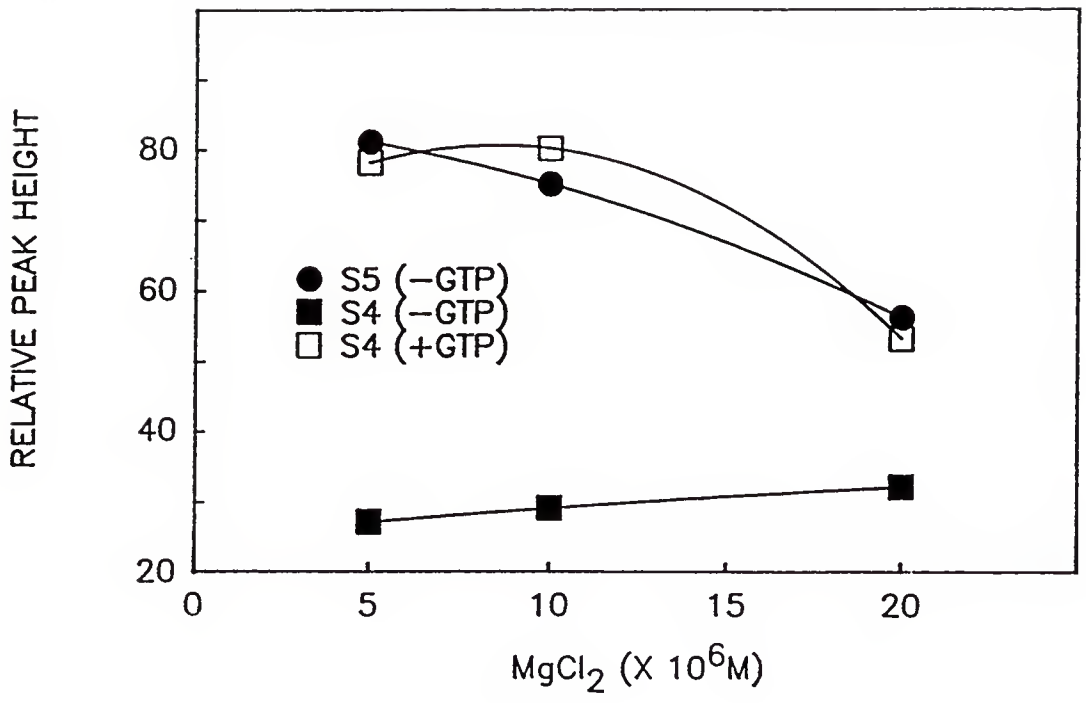


Figure 3-6 B Densitometric analysis of the autoradiogram in figure 3-6 A. The amount of radiolabeling of the 38 and 43 kDa ribosomal proteins was plotted versus the concentration of $MgCl_2$ in the reaction buffer. Note that no radiolabeling of the 38 kDa protein was detected in the presence of 5 μM GTP.

The three previous experiments suggest that the optimal ionic composition of the reaction buffer for the binding of GTP and its 8-Azido analog to 28S ribosomes was 100 mM KCl, 5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4, and 1 mM BME and will be referred to as reaction buffer A in this paper. The optimal uv photolabeling conditions were 300 $\mu W/cm^2$ for 10 min for 8-Azido GTP.

With these new conditions another attempt was made to analyze photolabeled ribosomal proteins by two dimensional PAGE. In figure 3-7 A to F, 28S ribosomes (1 μM) were photoaffinity labeled with increasing concentrations of [γ^{32}P]8-Azido GTP (0.3, 1, and 3 μM) in the presence and absence of excess (10 μM) GTP. These amounts of [γ^{32}P]8-Azido GTP correspond to 9, 20, and 60% saturation of the GTP binding site on the ribosome (based on a Kd of 1.92 μM). Small subunit ribosomes were resolved by 2D-PAGE and radiolabeled proteins were detected by autoradiography.

At all concentrations tested, S5 was specifically radiolabeled by [γ^{32}P]8-Azido GTP (figure 3-7 A to F) and the radiolabeling increased directly proportional to the concentration of [γ^{32}P]8-Azido GTP (figure 3-7 G). The presence of GTP completely blocked photoincorporation of the GTP analog into S5. S4 was also radiolabeled, but in a nonspecific manner, since excess amounts of GTP failed to block photoincorporation of [γ^{32}P]8-Azido GTP. These results support the tentative finding seen in the initial attempt to

Figure 3-7 Two dimensional PAGE analysis of photoaffinity labeled 28S ribosomes with increasing concentrations of [γ^{32}P]8-Azido GTP. Bovine 28S ribosomes (1 μM) were irradiated with uv light for 10 min at 300 μW/cm^2 on ice in the presence of increasing concentrations of [γ^{32}P]8-Azido GTP (0.3 to 3 μM). Results in the presence (B, D, & F) and absence (A, C, & E) of 10 μM GTP is also shown. The samples were irradiated with uv light for 10 min at 300 μW/cm^2 on ice and under dim red light. The specific activity of the [γ^{32}P]8-Azido GTP was 2.9 Ci/mmol just prior to use. Silver stained gels are on the left and their corresponding autoradiograms on the right. Autoradiograms were exposed for 7 days. The Mr markers used were the same ones described in figure 3-2. See Methods for additional experimental details.

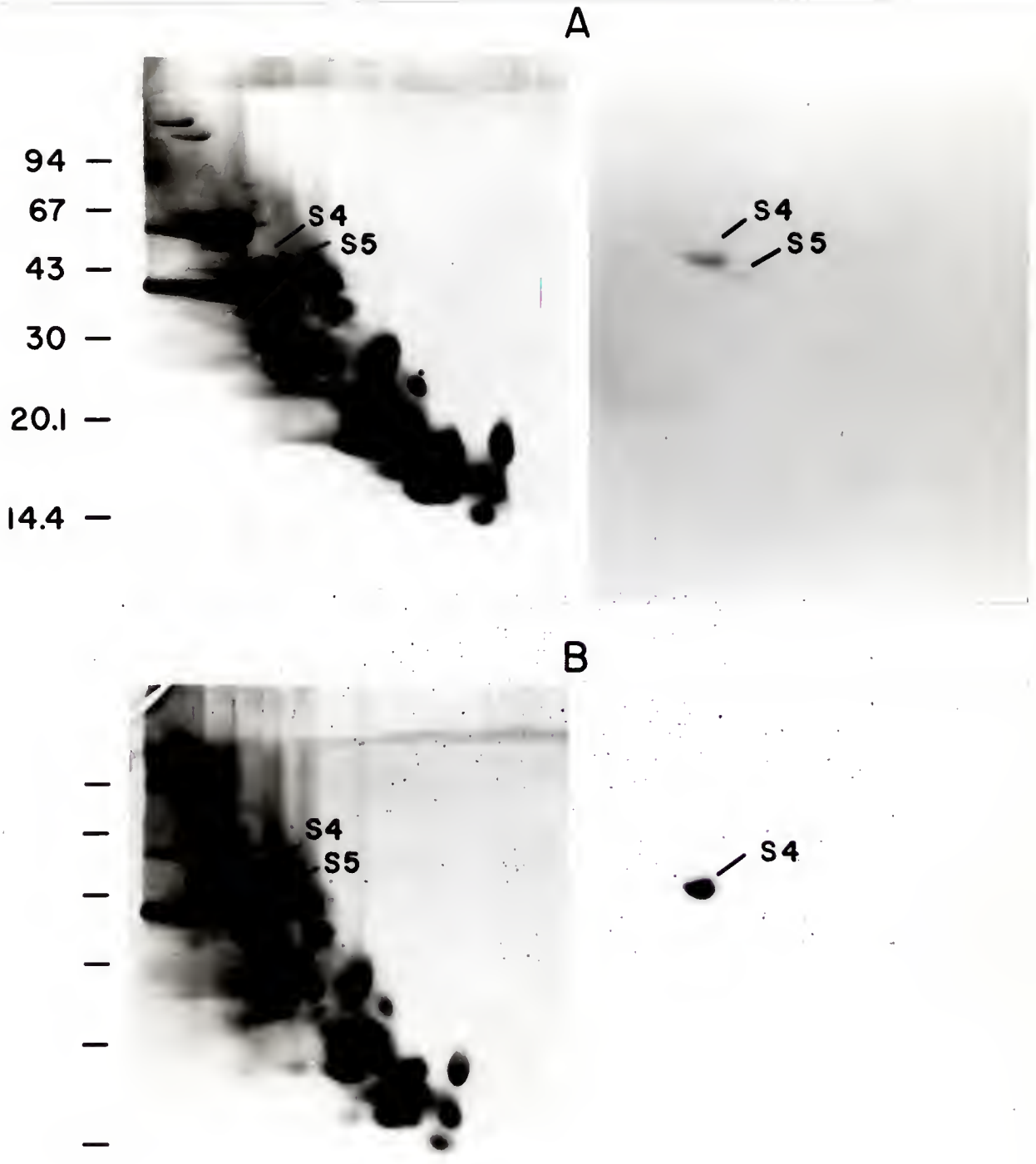


Figure 3-7 A & B Two dimensional PAGE analysis of photoaffinity labeled 28S ribosomes with 0.3 μM (9% saturation) [γ^{32}P]8-Azido GTP (panels A & B). The presence of 10 μM GTP (panel B) blocked radiolabeling of S5.

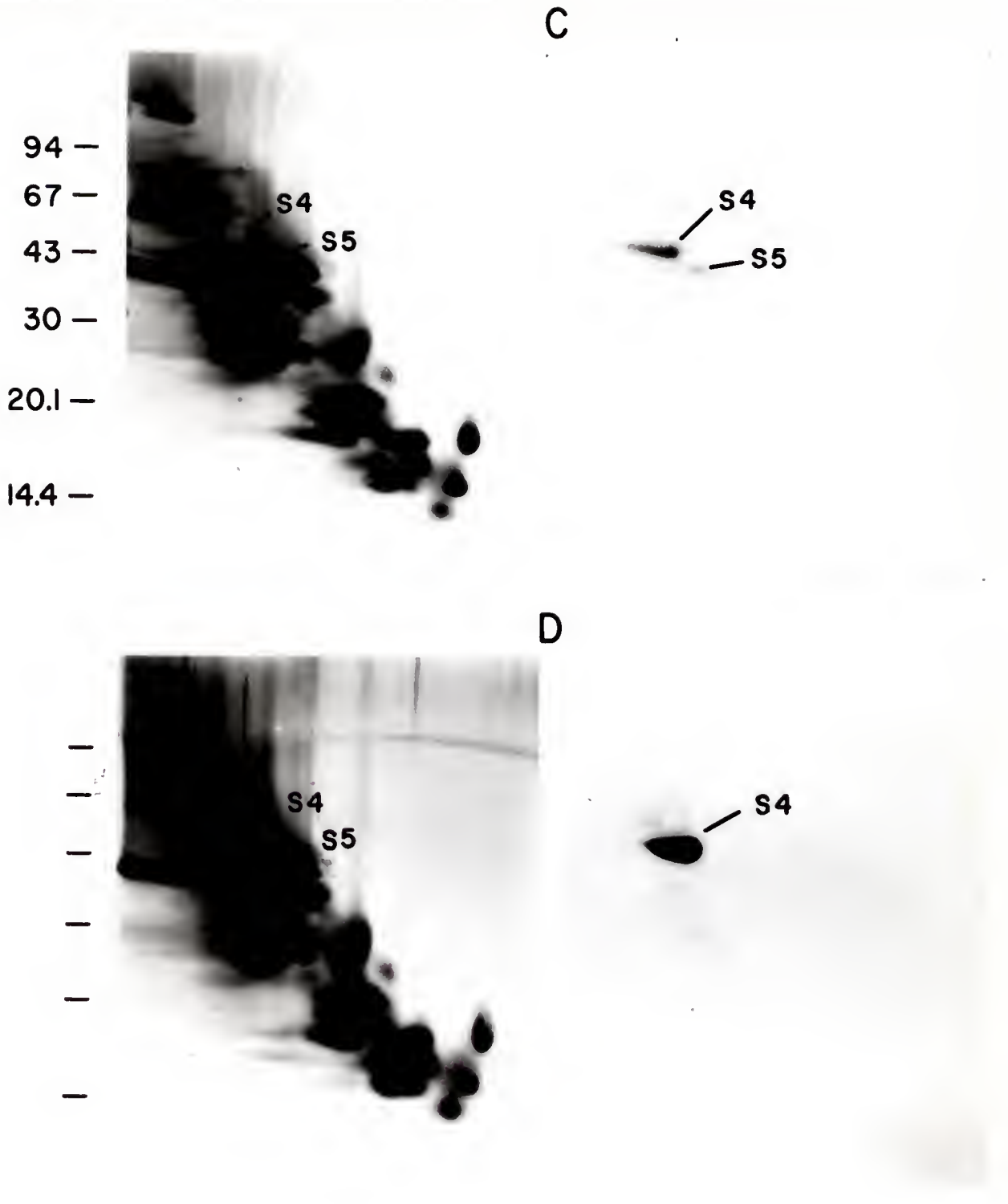


Figure 3-7 C & D Two dimensional PAGE analysis of photoaffinity labeled 28S ribosomes with 1.0 μM (28% saturation) [γ^{32}P]8-Azido GTP (panels C & D). Note, that in the presence of 10 μM GTP (panel D) S5 was not radiolabeled.

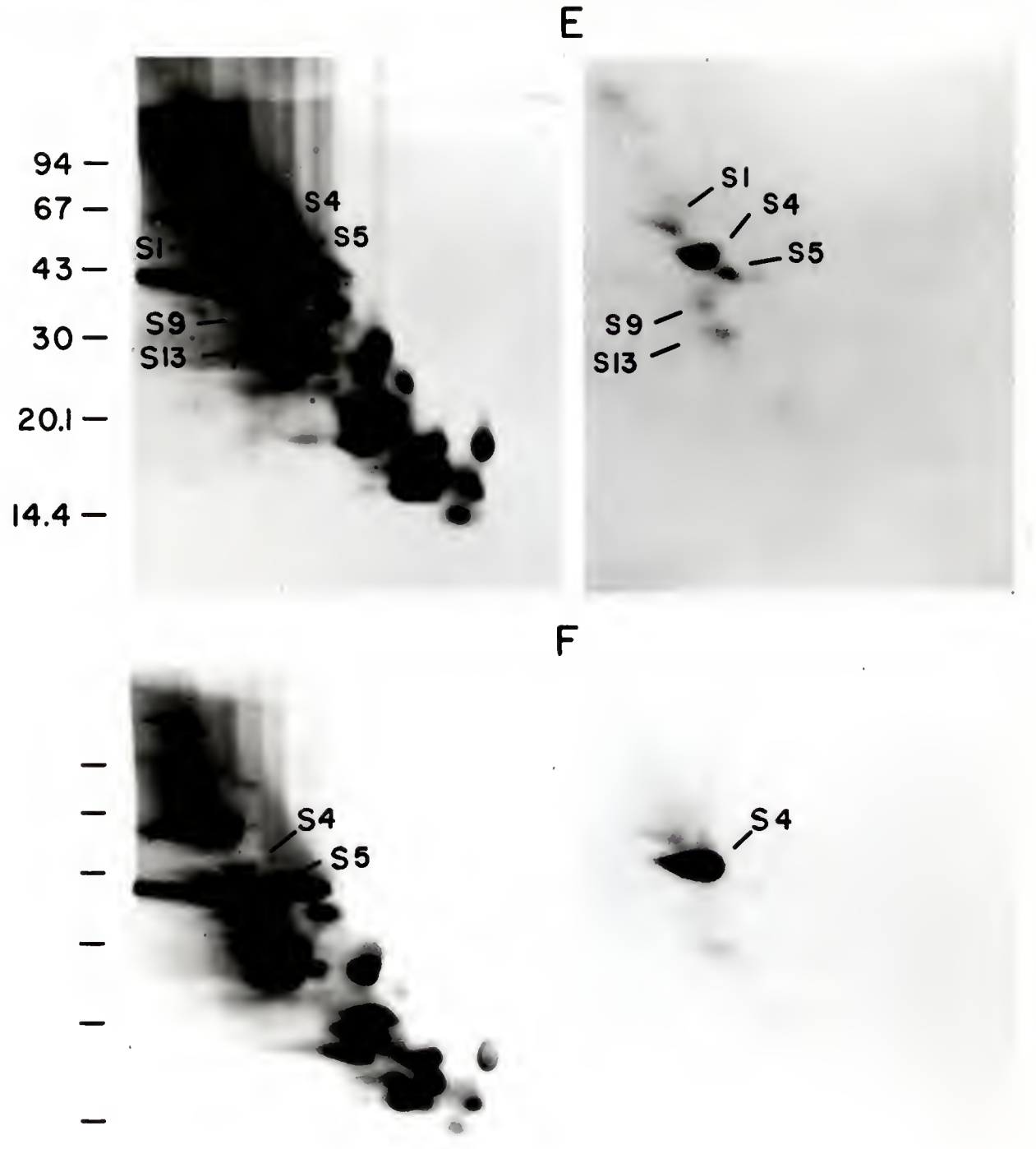


Figure 3-7 E & F Two dimensional PAGE analysis of photoaffinity labeled 28S ribosomes with 3.0 μM (56% saturation) [γ^{32}P]8-Azido GTP (panels E & F). Note, that in the presence of 10 μM GTP (panel F) S5 was not radiolabeled.

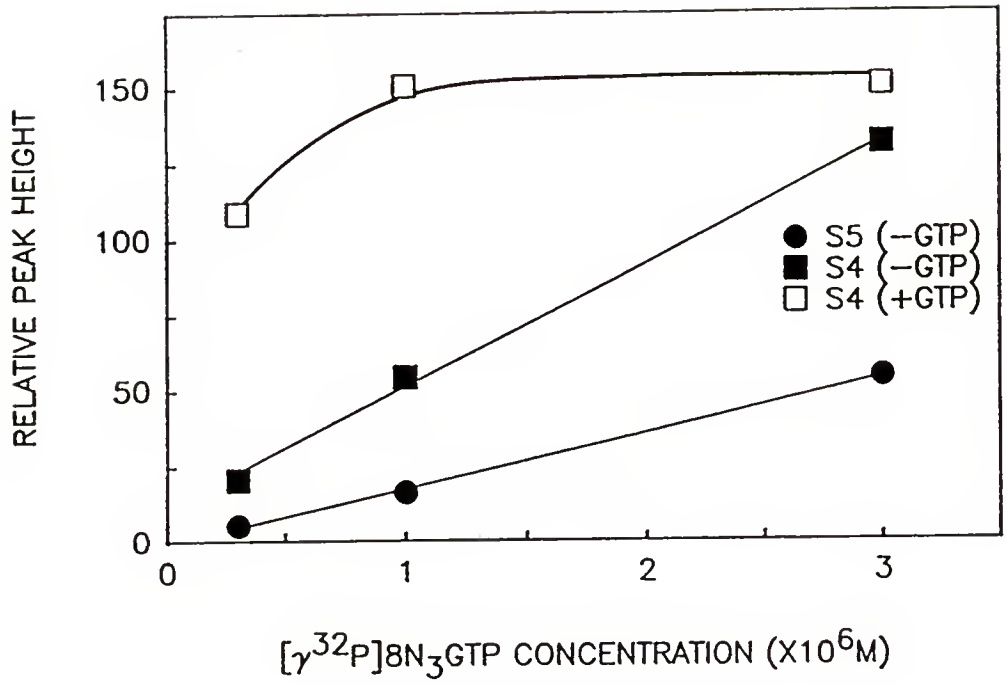


Figure 3-7 G Densitometric analysis of the GTP specific and nonspecific radiolabeling of ribosomal proteins S5 and S4 respectively. In panel G densitometric analysis of the autoradiograms in panels A to F for the specific labeling of S5 and nonspecific labeling of S4 is shown.

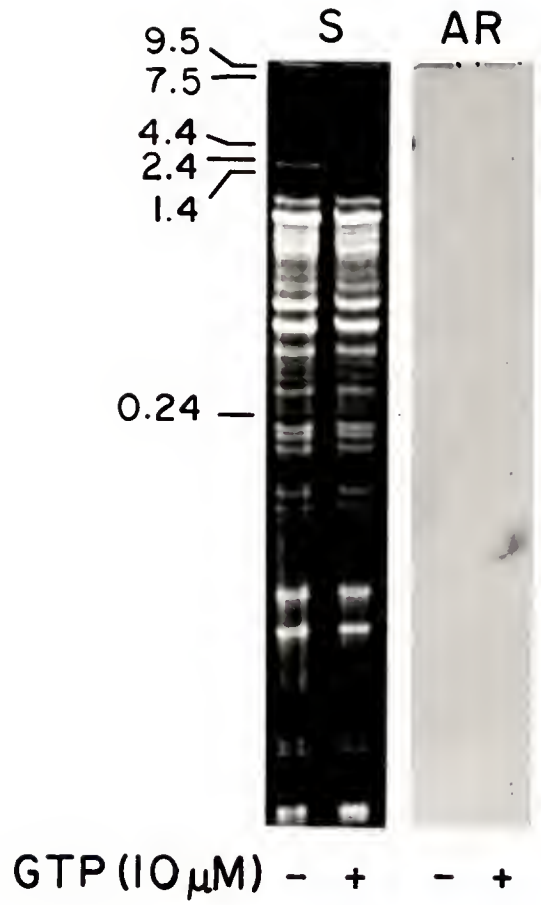


Figure 3-7 H Analysis of rRNA extracted from 28S ribosomes irradiated in the presence of [γ^{32}P]8-Azido GTP. This is an analysis of the rRNA extracted from photoaffinity labeled 28S ribosomes shown in figure 3-8, panels C & D (the result in all other panels was the same, no radiolabeling of rRNA). The rRNA was extracted following uv irradiation by 9M urea and 3M LiCl and electrophoresed by 1D urea PAGE (7.5% PAGE) as described in Methods. The left panel is a methylene blue stained gel (S) and its corresponding autoradiogram (AR) on the right. The autoradiogram was exposed for 41 days. The molecular weight markers are six synthetic poly(A)-tailed RNAs ranging in length from 0.24 to 9.49 kb (Bethesda Research Labs). No rRNA fragments were radiolabeled.

identify the GTP binding protein by 2D-PAGE analysis (figure 3-3), that S5 was specifically labeled by 8-Azido GTP. The development of more stringent ionic and milder photoirradiation conditions did not eliminate nonspecific labeling of S4. S4 was the most intensely labeled protein which increased proportionally with the concentration of [γ^{32}P]8-Azido GTP. S4 appears to be reciprocally labeled in the presence of excess amounts of GTP (figure 3-7 G). The amount of S4 labeling increases in the presence of 10 μM GTP, even though the radionucleotide was diluted as much as 33 fold. This suggests this site may be a lower affinity site for 8-Azido GTP. No radiolabeling of 12S rRNA was detected (figure 3-7 H), suggesting no direct involvement of rRNA in binding of 8-Azido GTP to 28S ribosomes.

Discussion

Photoaffinity labeling of 28S ribosomes with 8-Azido GTP revealed that a single 38 kDa ribosomal protein bound the GTP analog in a specific manner (figure 3-2 to 3-7). Unlabeled GTP could displace the radiolabeling of this protein suggesting the site was specific for GTP. A 43 kDa protein was also radiolabeled but the labeling was nonspecific because excess amounts of GTP failed to block binding. The amount of radiolabeling on the 38 kDa protein increased with increasing concentrations of [α^{32}P]8-Azido GTP and was saturable (figure 3-2 B) in a fashion consistent with the binding of this compound to 28S ribosomes as determined by filter binding

assays (figure 2-3). The photolabeling reaction was done under the same ionic conditions (reaction buffer B) as were used in filter binding assays to assess [α^{32}P]8-Azido GTP binding to 28S ribosomes.

The identity of this labeled protein was examined by 2-dimensional PAGE. Two proteins retained the label, one S4, was the predominately radiolabeled protein but the labeling was nonspecific because it was not diminished in the presence of excess amounts of GTP. The second protein, S5, was specifically radiolabeled by [α^{32}P]8-Azido GTP. Excess concentrations of GTP blocked binding to this protein suggesting that it is the specific binding site for GTP.

Comparing these results to those of the 1-dimensional analysis (figure 3-2), the 43 kDa nonspecifically labeled protein correlates to S4 in both Mr and binding specificity, and the 38 kDa protein would be S5 for the same reasons. The only disparity was that the radiolabeling of S5 was less than that seen on S4 in the two-dimensional PAGE analysis (figure 3-7). The disparity likely resides in differences in sample preparation. In two-dimensional PAGE analysis, photolabeled 28S ribosomes are extracted under acidic conditions (pH 2.5) to remove rRNA prior to separation in the first dimension in urea also under acidic conditions (pH 4.3). Temple et al. (1966) and Maliarik and Goldstein (1988) have observed that some covalent bonds formed by the aryl (purine) nitrene adducts are acid labile. In previous attempts to resolve

photolabeled ribosomes with the azido analog no radiolabeled proteins were detected. Increasing the pH of the extraction step to neutrality, only one radiolabeled protein, S4, was found and it was not blocked in the presence of GTP (data not shown). However when the pH of the first dimensional separation was raised from 4.3 to 5.0 we were able to observe not only the nonspecific labeling of S4 but also specific labeling on S5 (figure 3-3 and 3-7). The separation of these basic ribosomal proteins in the first dimension in urea could not be done at any higher pH without risking alteration of the relative mobilities of the proteins in a defined system (Matthews et al.,1982). Never-the-less, under the elevated pH conditions during sample preparation and analysis, it is clear that S5 is the protein binding GTP in a specific manner. Concerns about the survivability of the covalent bound formed by the nitrene adduct, and photolabeling under more physiological ionic conditions lead to studies to determine the minimum photoirradiation required and optimal ionic conditions for this reaction.

The minimum fluency ($\mu W/cm^2$) and duration of irradiation to achieve labeling of ribosomal proteins using 254 nm light with 8-Azido GTP, were 300 $\mu W/cm^2$ and 10 min, respectively (figure 3-5). This was determined over the span of two experiments in which both the [$\alpha^{32}P$] and [$\gamma^{32}P$]8-Azido GTP were used, with no differences seen in the pattern of radiolabeled proteins. O'Brien et al. (personal communication) found that

binding of [γ^{32}P]GTP to 28S ribosomes, under conditions similar to those used here, resulted in no hydrolysis of the [γ^{32}P] label even after several hours of incubation with the ribosome. This indicates that under these binding conditions [γ^{32}P]8-Azido GTP can be used with no hydrolysis of the [γ^{32}P] to obscure the results of photoaffinity labeling.

The length of time required (10 min) for adequate levels of radiolabeling are somewhat longer than reported by others (typically 2 to 6 min at fluency < 1000 $\mu W/cm^2$ on ice in various buffer biological solutions, see Potter and Haley, 1983). However, irradiation for 4 min at 300 W/cm^2 was minimally acceptable in 1-dimensional systems, but due to greater concentrations of protein (1 versus 0.5μM ribosomes) it was not judged adequate for 2-dimensional analysis.

Several enzymatic systems which were subjected to photoaffinity labeling saw no appreciable loss in function when irradiated with 254 nm light at low temperatures for as long as 10 min if the fluency remained below 800 W/cm^2 (Potter and Haley, 1983). These guidelines were used, since no functional assay exist for the 28S ribosome which could be used to monitor uv induced damage.

Geahlen and Haley (1977) reported that the half-life of the 8-azido GMP (160 μM at neutral pH) was approximately 20 sec in protein free buffer solution at approximately 1000 $\mu W/cm^2$ with 254 nm light. Since only minute quantities of the 8-azido GTP were available, it was not possible to examine the

uv degradation of the 8-azido to N_2 and 8-NH_2-GTP by spectrophotometric means as done by Geahlen and Haley (1977). Also, no suitable thin layer chromatography or other method could be found that could discriminate between 8-azido and the 8-NH_2 GTP though it was determined that the gamma phosphate was not lost following irradiation under the conditions used in this study (data not shown).

Efforts to develop more physiologically relevant ionic conditions for GTP binding to 28S ribosomes focused upon two components of the reaction buffer, $MgCl_2$, and NH_4Cl. In the initial binding experiments of this study the ionic composition of the reaction buffer (B) was adopted from the work of Bodley (Bacca, et al., 1976) who developed it to study GTP binding to bacterial EF-G. The concentration of the divalent cation, $MgCl_2$ (20 mM), was reduced to 5 mM to alleviate concerns about subunit dimerization which could alter GTP binding to the ribosome. O'Brien (1971) showed that the mitochondrial monoribosomes are stabilized at the higher concentration of $MgCl_2$ (20 mM $MgCl_2$ in 100 mM KCl) or greater. This occurs because divalent cations form salt bridges which stabilize rRNA interactions between subunits which results in subunit association or aggregation. At 5 mM $MgCl_2$, mitoribosomes subunits exist as discrete particles. GTP-Mg^{+2} binds as a complex to GTP-binding proteins. The Mg^{+2} ion binds tightly and nM concentrations of the divalent cation commonly suffice to fulfill this requirement (Gilman, 1987). Though

only modest increases in the photoincorporation of [γ^{32}P]8-Azido GTP in 28S ribosomes were observed at 5 mM $MgCl_2$ (figure 3-6), this reduced concentration of the divalent cation was adopted.

The replacement of the monovalent cation NH_4^+ by K^+ was examined because NH_4Cl is known to be toxic to the peptidyl transferase activity of the mitoribosome large subunit (Denslow and O'Brien, personal communication). Though we are studying a nucleotide binding activity on the small subunit it was deemed prudent to replace this monovalent cation in the event NH_4Cl is also toxic to some function of the small subunit, and in the event that future studies of peptidyl transferase activity might be undertaken in conjunction with this GTP-binding activity. Additionally, we wanted to increase the ionic strength to determine if GTP-binding would be affected by increasing the stringency to more physiologically relevant conditions. This was accomplished by replacing NH_4Cl with KCl and testing the effect of higher concentrations of KCl, equivalent to physiological concentrations (70 to 100 mM). GTP-binding activity was essentially unaltered at 100 mM KCl when compared to binding activity at 10 mM NH_4Cl (table 3-1). This indicated that GTP can bind the 28S ribosome even at moderate salt concentrations suggesting the binding is not predominately electrostatic in nature. Having established new ionic conditions (reaction buffer A) photoaffinity labeling of 28S ribosomes by

[γ^{32}P]8-Azido GTP was examined again. Photoaffinity labeling with increasing concentrations of 8-Azido GTP to a fixed concentra-tion of the ribosome, resulted in radiolabeling of the same proteins seen previously. S4 was nonspecifically radiolabeled and S5 was specifically radiolabeled by [γ^{32}P]8-Azido GTP. At very high saturating concentrations of the GTP analog, other ribosomal proteins were also labeled in a nonspecific manner (figure 3-7 E). At all concentrations of [γ^{32}P]8-Azido GTP used, S5 radiolabeling was blocked in the presence of excess GTP and the radiolabeling of S5 increased with increased concentrations of the [γ^{32}P]8-Azido GTP. Nonspecific radio-labeling of S4 also increased in a manner comparable to that seen for S5 but interestingly S4 radiolabeling increased in the presence of excess GTP and appeared to be saturable (figure 3-7 G). This suggested that S4 bound some of the [γ^{32}P]8-Azido GTP displaced from the binding site by GTP (10 μM) even though the specific activity of the radiolabeled GTP analog was decreased by 33 to 3.3 fold. This suggests that S4 binds GTP in a manner different than for S5, and is likely to have a lower affinity binding site.

CHAPTER 4

PHOTOAFFINITY LABELING OF 28S RIBOSOMES BY [α^{32}P]GTP

Introduction

Direct photoaffinity labeling of phosphofructokinase (Ferguson, and MacInnes, 1980) and tubulin (Nath, et al., 1985; Hesse, et al., 1987;and Linse and Mandelkow, 1988) with radiolabeled cAMP and GTP/GDP, respectively, encouraged us to explore the use of the [α^{32}P]GTP to photolabel the 28S ribosome. The chemical structure at the site of covalent attachment for these purines is not known, but the fact that maximal photolabeling of phosphofructokinase occurred at a wavelength equivalent to its absorption maxima suggests that the adenine portion of the nucleotide is the light sensitive chromophore (Ferguson and MacInnes, 1980). The use of the natural ligand takes advantage of the increased binding affinity thereby circumventing difficulties encountered due to lowered binding affinities of the 8-Azido GTP analog for the 28S ribosome (table 2-3). The disadvantages are that the mechanism of photoactivation is unknown and therefore we do not know if covalent bond formation is as nonselective a process as was the case for the nitrene. Another disadvantage is that the minimal quantum yield of product would be lower than for the

nitrene. In the case of cAMP, photolabeling of phosphofructokinase was reported to be approximately 0.01% (Ferguson and MacInnes, 1980) several hundred times less than for the nitrene. However that difficulty can be overcome to some degree because [α^{32}P]GTP can be obtained at specific activities as much as 300 to 600 times greater than [α^{32}P]- or [γ^{32}P]8-Azido GTP, respectively.

In this study we report successful photoaffinity labeling of 28S ribosomes with [α^{32}P]GTP. The photolabeling of ribosomal proteins was equivalent to that seen for [α^{32}P]- and [γ^{32}P]8-Azido GTP, confirming that S5 is the specific site for photoincorporation of GTP.

Materials and Methods

Reagents

[α^{32}P]GTP, and ATP and [γ^{32}P]ATP in triethylammonium salt were purchased from Amersham at specific activities of 410 and >3000 Ci/mmole, respectively. All unlabeled nucleotides were purchased from Sigma Chemical Co. as Type I reagents as described above.

Solutions

The same solutions as described above, in 8-Azido GTP photoaffinity labeling experiments, were used in these studies.

Methods

The same methods as described above, in 8-Azido GTP photoaffinity labeling experiments, were used in these studies.

Results

Photoaffinity Labeling of 28S Ribosomes with [α^{32}P]GTP

In an effort to substantiate the above findings photolabeling of small subunits with [α^{32}P]GTP was attempted. Hesse et al. (1987) successfully photolabeled ß-tubulin with [α^{32}P]GTP suggesting the natural ligand could be used to photoaffinity label the GTP binding ribosomal protein.

First, photolabeling conditions were varied to optimize photolabeling. 28S Ribosomes (0.5 µM) were photoaffinity labeled in presence of 0.5 µM [α^{32}P]GTP, which corresponds to 80% saturation (calculation based on a Kd of 15.2 nM) of the GTP binding site. Ribosomes were photolabeled for 2 and 10 min at increasing intensities (800, 1500, 2000, and 2500 µW/cm^2) of uv light (254 nm) on ice and under dim room light.

The pattern of radiolabeling of 28S ribosomes by [α^{32}P]GTP (figure 4-1) was similar to that for [α^{32}P]8-Azido GTP (figure 3-4) and [γ^{32}P]8-Azido GTP (figure 3-5). The 38 kDa ribosomal protein was specifically labeled by GTP because excess amounts of GTP blocked labeling of that protein. The 43 kDa ribosomal protein was still nonspecifically labeled in the presence of excess amounts of GTP as seen with the 8-Azido analog.

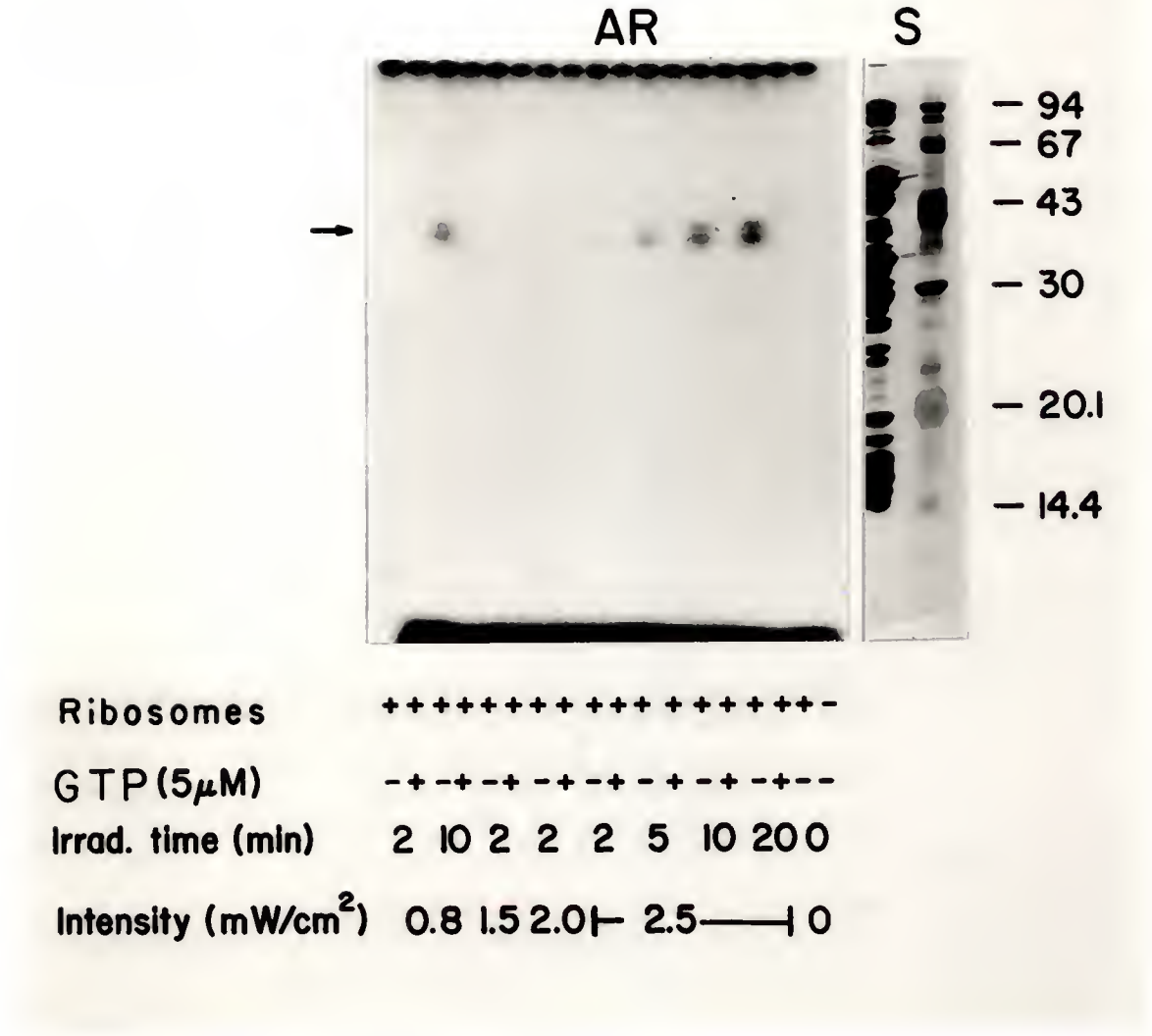


Figure 4-1 A Optimization of the intensity and duration of uv irradiation for photoaffinity labeling of 28S ribosomes with [α^{32}P]GTP. 28S ribosomes (0.5 μM) were incubated with 0.5 μM [α^{32}P]8-Azido GTP (80% saturation) in reaction buffer B. The samples were irradiated with uv light for 5 min at 800 μW/cm^2, on ice, prior to electrophoresis. [α^{32}P]GTP was diluted 10 fold with GTP to a specific activity of 32 Ci/mmol just prior to use. In panel A is a representative lane of silver stained (S) ribosomal proteins on the right and the corresponding autoradiogram (AR) on the left. The AR was exposed for 24 h before development. The presence and absence of ribosomes and unlabeled GTP as well as the intensity and duration of uv (254 nm) irradiation are noted below the autoradiogram. The arrow indicates the radiolabeled 38 kDa protein that is specifically labeled by GTP.

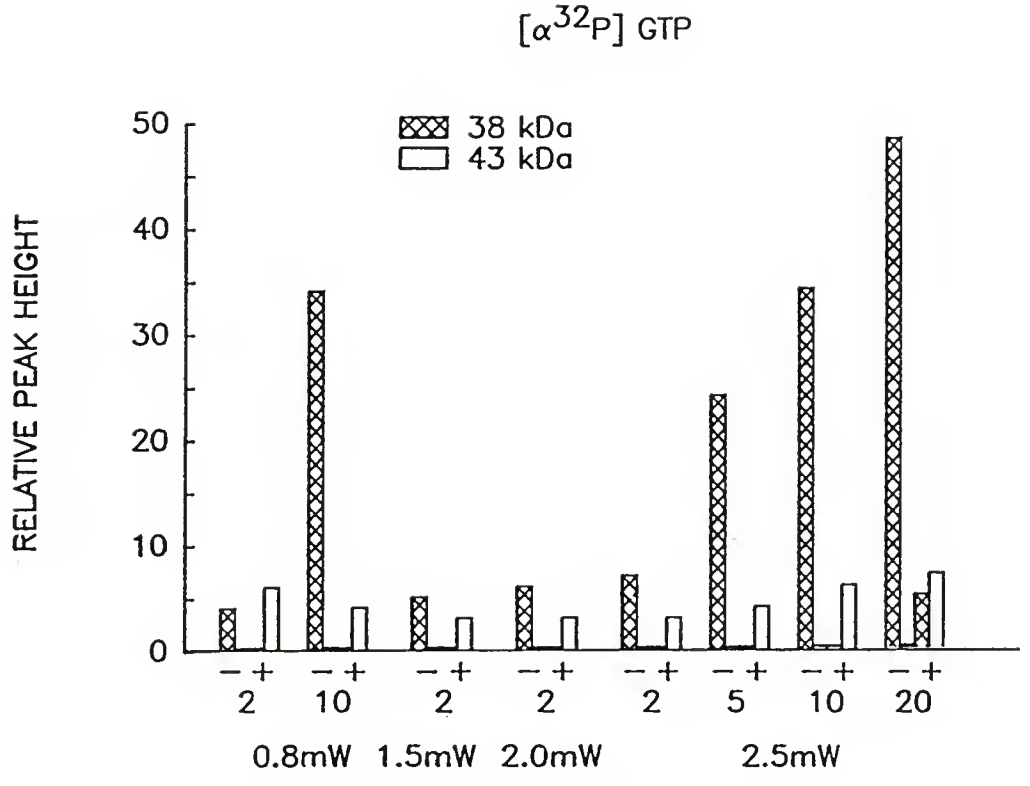
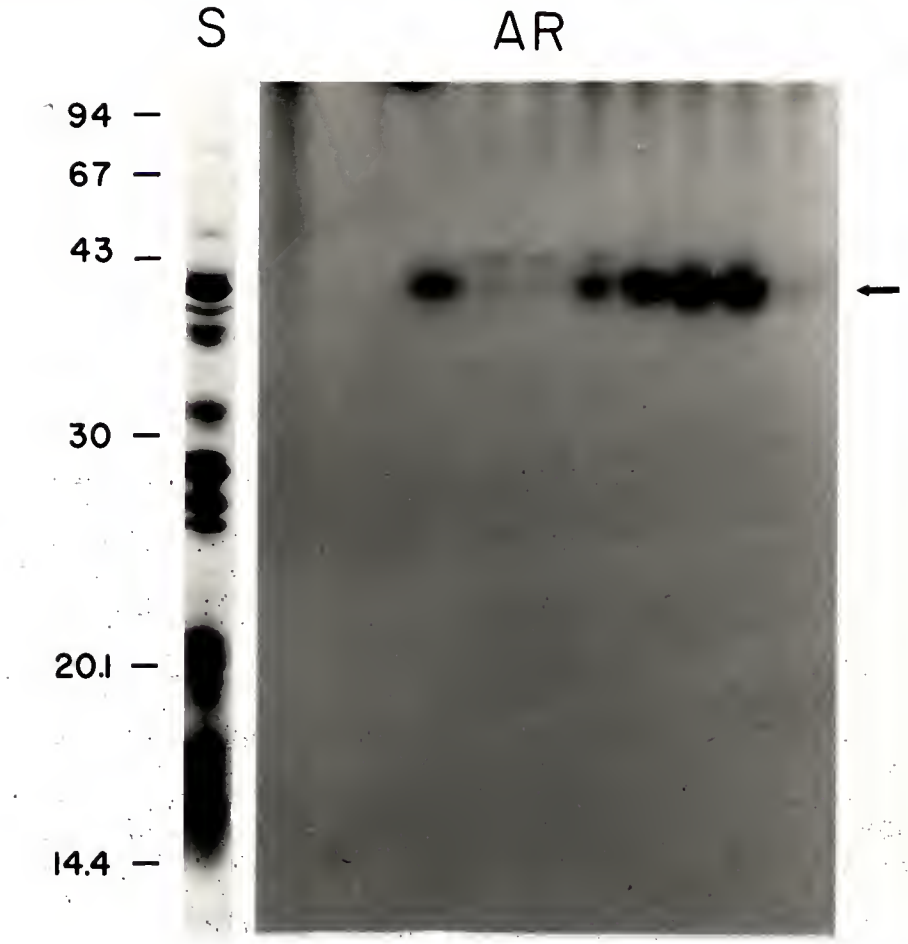


Figure 4-1 B Densitometric analysis of the autoradiogram shown in figure 4-1 A. Densitometric analysis was done for the specifically labeled ribosomal protein (38 kDa) and the nonspecifically labeled protein (43 kDa) ribosomal protein at varying intensities and duration of uv irradiation. In the presence (+) of 5 μM GTP, the 38 kDa protein was not radiolabeled. The duration and intensity of uv irradiation is indicated below the figure. For additional experimental details see Methods.

Photolabeling of the 38 kDa protein by [α^{32}P]GTP was more dependent on the duration of irradiation than the intensity of the uv light (figure 4-1 B). No differences were seen by densitometric analysis in the amount of photolabeling of the 38 kDa protein when the intensity of uv irradiation was increased from 800 to 2500 $\mu W/cm^2$ for 2 min. However the amount of photolabeled 38 kDa protein was dramatically increased nearly 10 fold when the duration of irradiation was lengthened to 10 min at 2500 $\mu W/cm^2$. The amount of photolabeling at 800 and 2500 $\mu W/cm^2$ was in fact nearly identical for irradiation at 10 min. This data suggests that irradiation for 5 min at 800 $\mu W/cm^2$ would produce equivalent amounts of radiolabeling to that seen for 5 min at 2500 $\mu W/cm^2$ (figure 4-1 B). The amount of radiolabeling in that case was sufficient for photoaffinity labeling of 28S ribosomes so uv (254 nm) irradiation at 800 $\mu W/cm^2$ for 5 min was adopted for subsequent photolabeling experiments with [α^{32}P]GTP.

To determine the extent of binding specificity of the 28S ribosome several nucleotide tri- and diphosphates were tested for their ability to block photoaffinity labeling of 28S ribosomes by [α^{32}P]GTP. Of the nucleotides tested, only GTP and GDP, and to a much lesser extent, GMP, were able to block photolabeling of the 38 kDa ribosomal protein by [α^{32}P]GTP (figure 4-2 A). These findings agree with the results of filter binding assays (figure 2-2) and photoaffinity labeling experiments with 8-Azido GTP, that there is a single GTP

Figure 4-2 A Specificity of the photoinduced radiolabeling of 28S ribosomal proteins by [α^{32}P]GTP in the presence of other nucleotides. Ribosomes small subunits (0.5 μM) were incubated in the presence of 0.28 μM [α^{32}P]GTP (50% saturation) in the presence (+)and absence (-) of 5 μM unlabeled nucleotide (GTP, GDP, GMP, ATP, CTP, UTP, and GTP + ATP). The samples were irradiated with uv light for 5 min at 800 μW/cm^2 on ice, in reaction buffer A. In panel A a representative silver stained lane of 28S ribosomes is shown on the left and the corresponding autoradiogram (AR) on the right. The presence and absence of ribosomes, uv irradiation, [α^{32}P]GTP, and unlabeled nucleotide is shown under the AR. The specific activity of the [α^{32}P]GTP was 410 Ci/mmol and the autoradiogram was exposed for 24 hours. The arrow identifies the 38 kDa protein specifically radiolabeled by GTP.



Ribosomes	−	+	+	+	+	+	+	+	+	+	+
UV	+	+	−	+	+	+	+	+	+	+	+
[α^{32}P]GTP	+	−	+	+	+	+	+	+	+	+	+
GTP	−	−	−	−	+	−	−	−	−	−	−
GDP	−	−	−	−	−	+	−	−	−	−	−
GMP	−	−	−	−	−	−	+	−	−	−	−
ATP	−	−	−	−	−	−	−	+	−	−	−
CTP	−	−	−	−	−	−	−	−	+	−	−
UTP	−	−	−	−	−	−	−	−	−	+	−
GTP+ATP	−	−	−	−	−	−	−	−	−	−	+

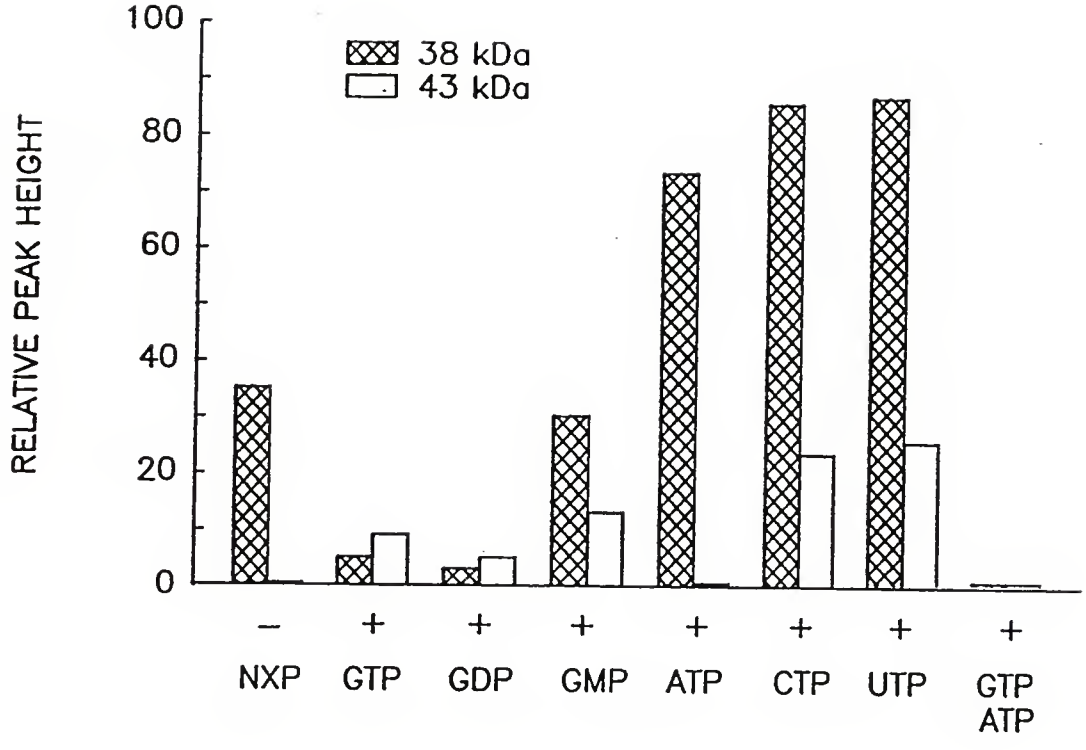


Figure 4-2 B Densitometric analysis of the autoradiogram (panel A) of 28S ribosomes photoaffinity radiolabeled with [α^{32}P]GTP in the presence (+) and absence (-) of various nucleotides. The analysis was done for both the specifically radiolabeled 38 kDa and the nonspecifically radiolabeled 43 kDa protein. In the presence of 5 μM GTP and GDP photoinduced radiolabeling of the 38 kDa protein by [α^{32}P]GTP was blocked, and 5 μM ATP blocked radiolabeling of the 43 kDa protein. See Methods for additional experimental details.

binding site on the 28S ribosome that is specific for GTP and GDP. The site for nucleotide binding is a 38 kDa ribosomal protein, identified as S5 by 2-dimensional analysis of 28S ribosomes photoaffinity labeled with 8-Azido GTP (figure 3-3 and 3-7).

The 43 kDa ribosomal protein was radiolabeled by [α^{32}P] GTP, except in the presence of ATP (figure 4-2 A). Densitometric analysis (figure 4-2 B) indicated that labeling of the 43 kDa protein was completely blocked in the presence of excess amounts of ATP suggesting for the first time that this site may specifically bind a nucleotide.

Millipore filter binding and binding inhibition assays (O'Brien et al., 1990; and figures 2-1 and 2-2) strongly indicated that a high affinity site that binds one molecule of GTP or GDP exists on the 28S ribosome. Autoradiographic analysis of one dimensional gels following photoaffinity labeling of 28S ribosomes suggest that a 38 kDa ribosomal protein is the specific GTP/GDP binding site.

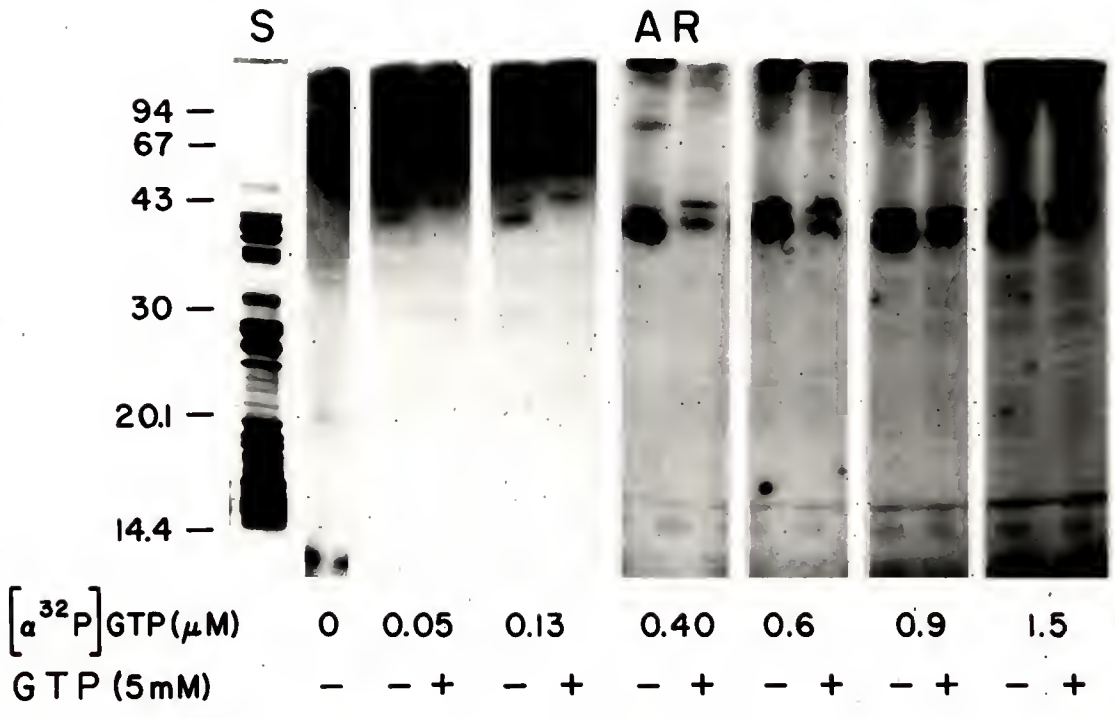
Photoaffinity labeling of this site results in a small but reproducible percentage of the site covalently labeled by [α^{32}P]GTP. Therefore analysis of the amount of radiolabeling of the 38 kDa ribosomal protein following photolabeling of 28S ribosomes with increasing concentrations of [α^{32}P]GTP would produce a binding curve similar to that seen in filter binding assays with intact small subunits if they represent the same binding activity.

Small subunits (0.5 μM) of mitochondrial ribosomes were photoaffinity labeled with increasing concentrations (0.05 to 1.5 μM) of [α^{32}P]GTP and in the absence and presence of 5 μM GTP in reaction buffer A as described above. Photolabeling was done at 800μW/cm^2 for 5 min on ice under dim room light. Following autoradiographic analysis (figure 4-3 A) of ribosomal proteins resolved on a 12% polyacrylamide Laemmli gel, the radiolabeled 38 and 43 kDa protein bands were excised and analyzed for the amount of bound [α^{32}P]GTP by ß-scintillation counting (figure 4-3 B, upper panel). The amount of radioactivity due to specific radiolabeling of the GTP binding site was determined by the difference in cpm found on the 38 kDa protein in the presence and absence of 5 μM GTP. This net radioactivity was converted to pmol of bound [α^{32}P]GTP based upon the calculated percent photoincorporation of the labeled GTP and its specific activity as discussed below. The pattern of radiolabeling was identical to previous studies.

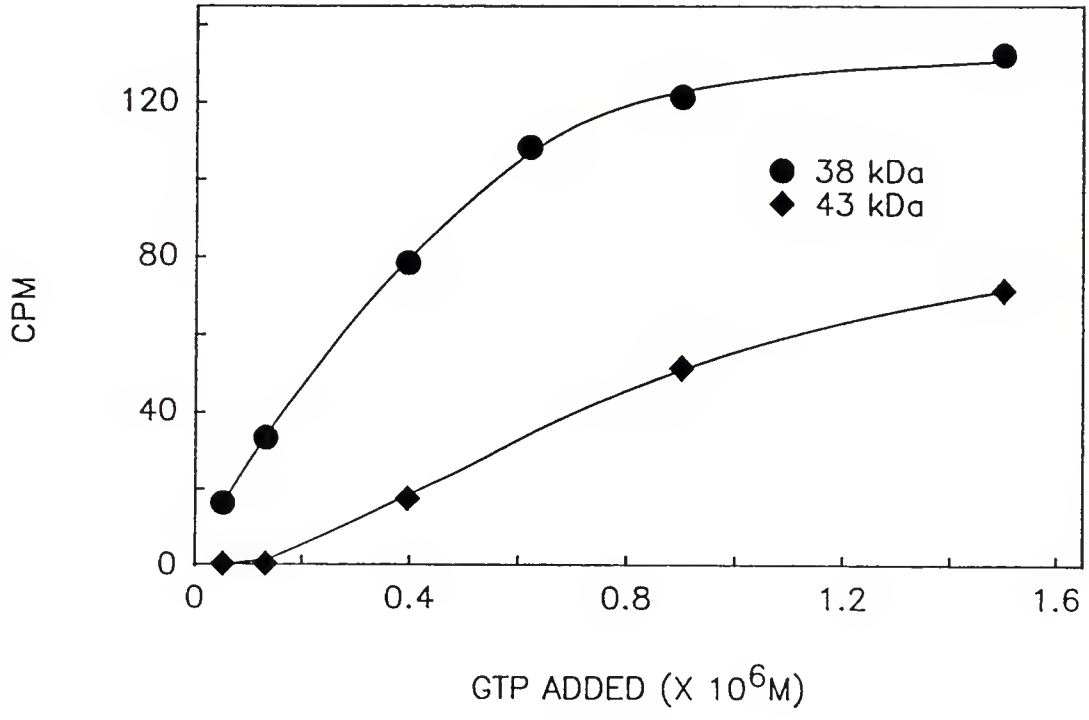
The amount of radiolabeling the 38 kDa protein did increase rapidly with increasing concentrations of [α^{32}P]GTP. At molar ratios of [α^{32}P]GTP to ribosomes greater than one, the radiolabeling of the 38 kDa protein did not increase further indicating saturation of the GTP binding site. Assuming that at saturation one molecule of GTP was bound to a ribosome the percent of photoincorporation of [α^{32}P]GTP into the 38 kDa protein was determined. At a specific activity of 116.4

Figure 4-3 A & B Determination of the binding affinity of [α^{32}P]GTP for the specifically labeled 38 kDa ribosomal protein by photoaffinity labeling of 28S ribosomes. 28S Ribosomes (0.5 μM) were incubated in reaction buffer A with increasing concentrations (0.05, 0.13, 0.4, 0.6, 0.9, and 1.5 μM) of [α^{32}P]GTP (corresponding to 10, 25, 70, 90, 120, and 200% saturation of the GTP binding site on the 28S ribosome) and in the presence (+) and absence (-) of 5 μM GTP. Samples were irradiated as described in figures 4-1 and 4-2. The specific activity of [α^{32}P]GTP was 278 Ci/mmol. In panel A a representative lane of silver stained (S) ribosomal proteins is shown on the left and the corresponding autoradiogram (AR) on the right. The concentration of the [α^{32}P]GTP and GTP shown below the autoradiogram. The arrow identifies the 38 kDa protein specifically radiolabeled by GTP. In panel B the amount of radioactivity found in the 38 kDa protein bands due to specific photolabeling of that protein was plotted versus the concentration of ligand added. The amount of specific radiolabeling of this protein was found by subtracting the radioactivity found in the 38 kDa gel plug in the presence of 5 μM GTP from that amount found in the same protein in the absence of 5 μM GTP. Additionally the amount of radioactivity found in 38 kDa protein due to dilution of [α^{32}P]GTP with GTP was also subtracted. The amount of [α^{32}P]GTP covalently bound to the 38 kDa protein was calculated based on the specific activity and percent photoincorporation as described in Results. Additionally, the amount of radiolabeling of the nonspecifically radiolabeled 43 kDa protein is shown. The amount of radioactivity found in the 43 kDa band in the absence of 5 μM GTP is plotted versus the concentration of [α^{32}P]GTP. The 43 kDa band was not specifically labeled by [α^{32}P]GTP therefore the amount of radioactivity found in the corresponding band in the presence of 5 μM GTP was not subtracted. However, the amount of [α^{32}P]GTP bound to the 43 kDa band was calculated as above, based on the same specific activity and percent photoincorporation as used for the 38 kDa protein.

A


S
AR
94
67
43
30
20.1
14.4
[α32P]GTP(μM) 0 0.05 0.13 0.40 0.6 0.9 1.5
GTP (5mM) – – + – + – + – + – + – +

B


120
80
40
0
CPM
38 kDa
43 kDa
0
0.4
0.8
1.2
1.6
GTP ADDED (X 10^6M)

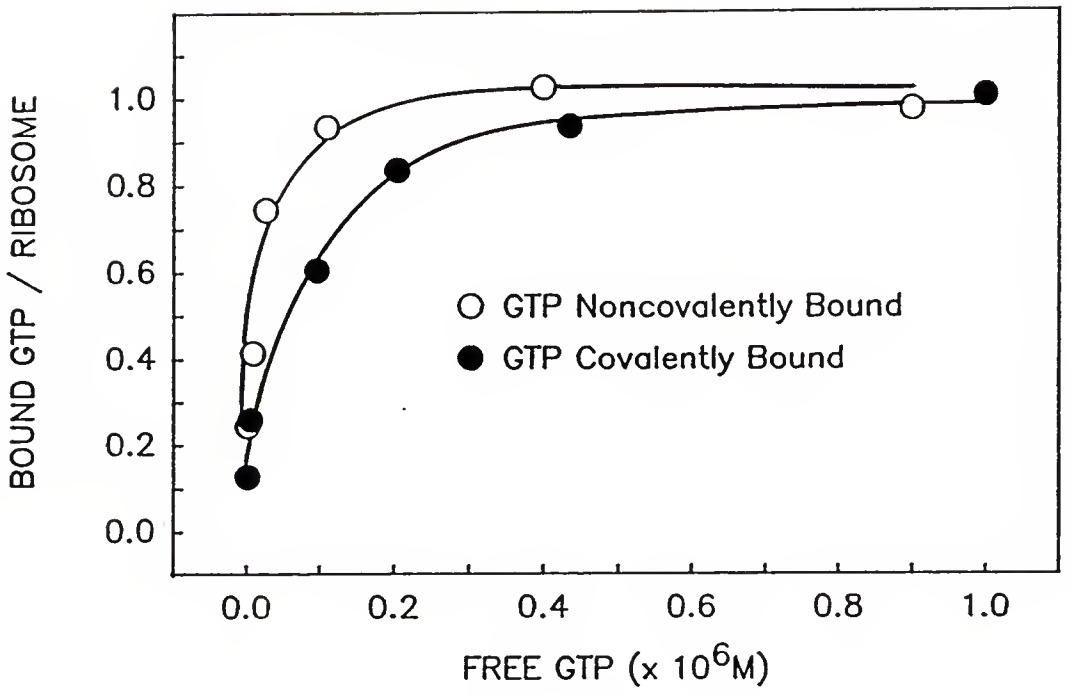


Figure 4-3 C Comparison of binding curves for photoinduced covalent binding of [α^{32}P]GTP to the 38 kDa ribosomal protein versus noncovalent binding to 28S ribosomes as determined by filter binding assays. The concentration of free [α^{32}P]GTP (calculated from data in panel B) was plotted versus that covalently bound to the ribosome in panel B. The binding of covalently bound [α^{32}P]GTP to the 38 kDa protein was compared to noncovalent binding of [α^{32}P]GTP to 28S ribosomes as determined by filter binding assays. The same preparation of ribosomes was used in both experiments. Scatchard analysis of both binding isotherms revealed apparent dissociation constants of 9.8 ± 2.4 and 42.6 ± 23.2 nM for covalent and noncovalently bound GTP, respectively. The number of binding sites for GTP on the ribosome for both experiments was 1.0.

Ci/mmole (or 225 cpm/fmol, at the time of analysis), 0.5 *f*mol of [α^{32}P]GTP was bound to 25 pmol of ribosomes when the binding site was saturated, for a percent photoincorporation of 2 X 10^{-3}%. The amount of covalently bound [α^{32}P]GTP was converted to concentrations of bound versus free ligand based upon the above specific activity and percent photoincorporation for [α^{32}P]GTP. Scatchard analysis of the resulting binding curve yielded an apparent dissociation constant and number of binding sites on the 28S ribosome of 42.6 ± 23.2 nM (kD ± SE) and 1.0, respectively (figure 4-3 C). This value is not significantly different from that obtained by filter binding assays (15.2 ± 8.64 nM, table 2-3) where radiolabeled GTP was bound to small mitochondrial ribosomal subunits. This suggests that the specific GTP binding site is the 38 kDa protein.

The radiolabeling of the 43 kDa protein with [α^{32}P]GTP was not blocked in the presence excess amounts of GTP. However radiolabeling of this protein did increase slowly as the concentration of [α^{32}P]GTP increased. The radiolabeling of the 43 kDa increased in a nearly linear manner suggesting a much lower affinity for [α^{32}P]8-Azido GTP for this protein.

Two dimensional PAGE analysis of 28S ribosomes photoaffinity labeled with [α^{32}P]GTP in the presence and absence of GTP and ATP was done to confirm the identity of the GTP binding protein. 28S Ribosomes (1.0 μM) were photo-affinity labeled (800μW/cm^2 for 5 min on ice) with 0.53 μM

Figure 4-4 Two dimensional PAGE analysis of 28S ribosomes photoaffinity labeled with [α^{32}P]GTP. Bovine 28S ribosomes (1 μM) were irradiated in the presence of 0.53 μM [α^{32}P]GTP (50% saturation, all panels) and 10 μM GTP (panel B), or 10 μM ATP and GTP (panel D). The samples were irradiated with uv light for 5 min at 800 μW/cm^2 on ice and under dim light. The specific activity of the [α^{32}P]GTP was 336 Ci/mmol. Silver stained gels (S) are on the left and their corresponding autoradiograms (AR) on the right. Autoradiograms were exposed for 4 days. The molecular weight markers used were the same ones described in figure 3-2. See Methods for additional experimental details.

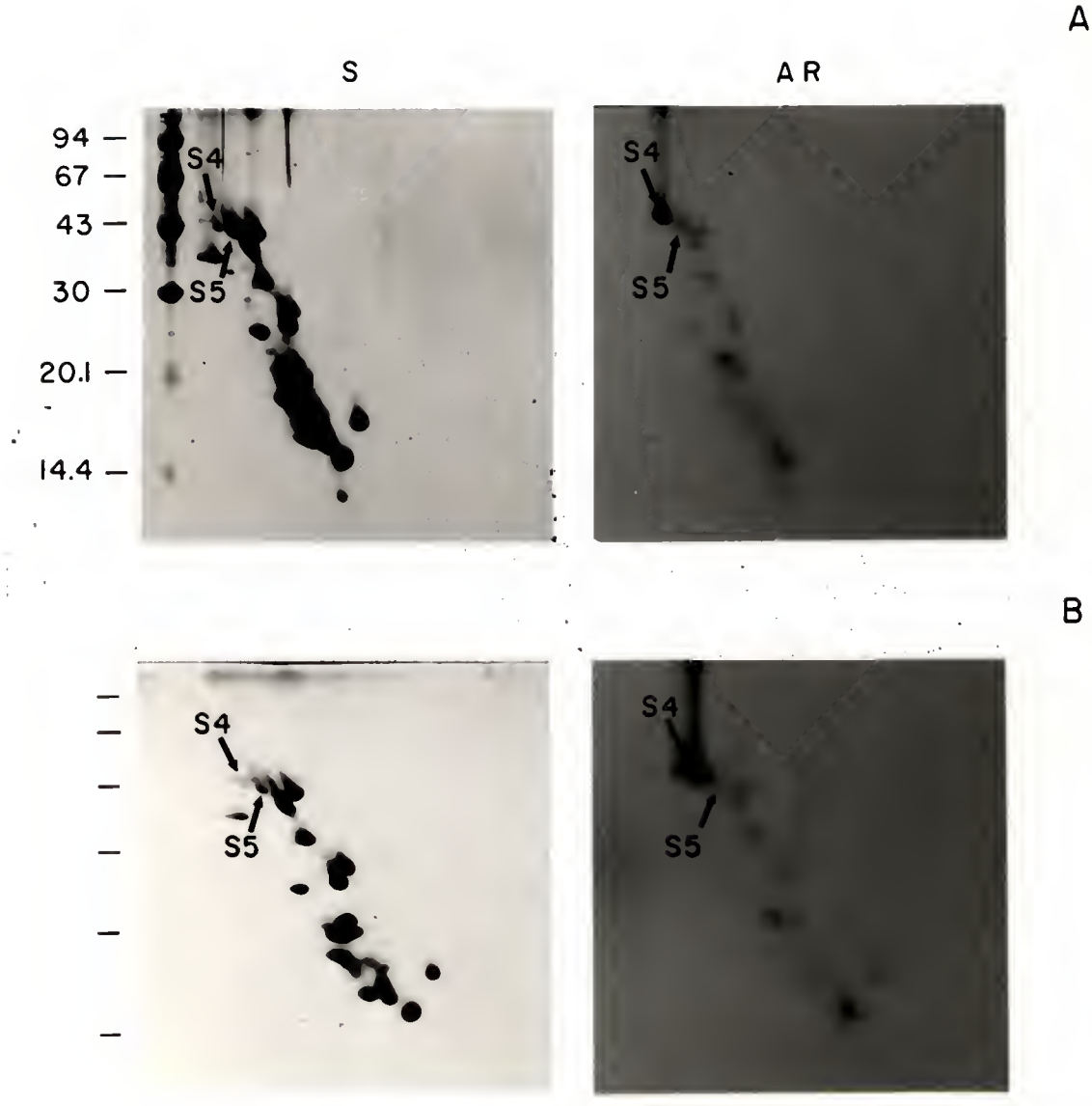


Figure 4-4 A & B Two dimensional PAGE analysis of 28S ribosomes photoaffinity labeled with [α^{32}P]GTP. Ribosomes were incubated and uv-irradiated with [α^{32}P]GTP in the absence (panel A) and presence (panel B) of 10 μM GTP. In the presence of GTP radiolabeling of S5 was blocked.

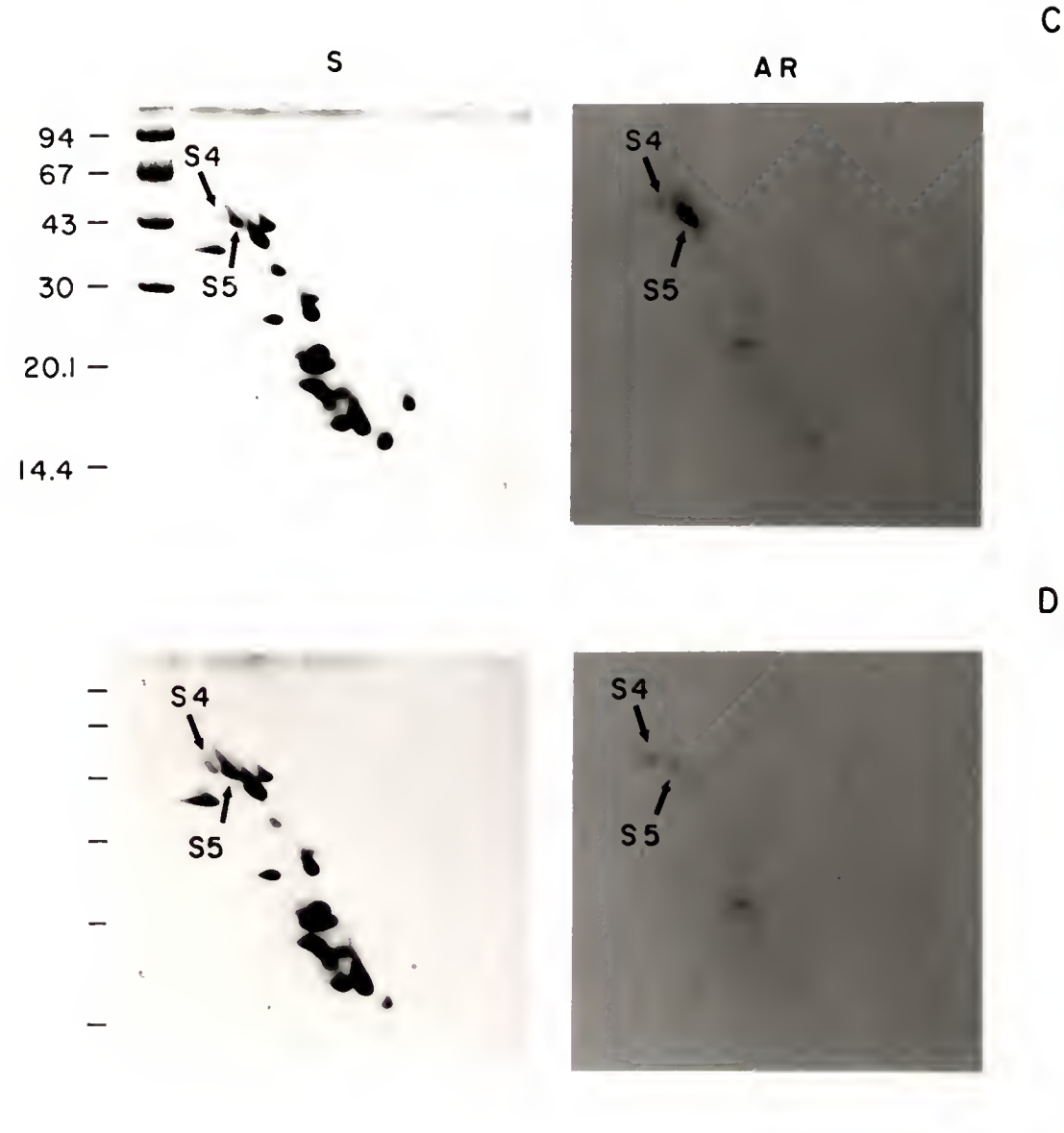


Figure 4-4 C & D Two dimensional PAGE analysis of 28S ribosomes photoaffinity labeled with [α^{32}P]GTP. Ribosomes were incubated and uv-irradiated with [α^{32}P]GTP in the presence of 10 μM ATP alone (panel C) and presence of 10 μM ATP and GTP (panel D). In the presence of ATP radiolabeling of S4 was blocked and with the addition of GTP, radiolabeling of S5 was also blocked.

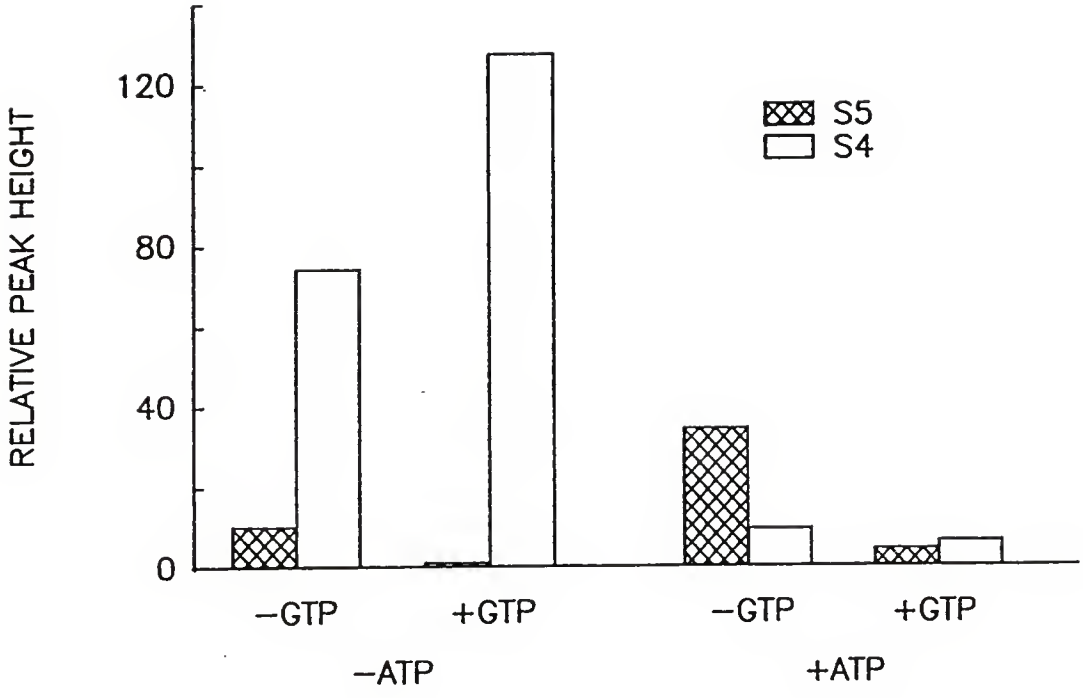


Figure 4-4 E Densitometric analysis of the 2D-PAGE autoradiograms (panels A to D) for the specific labeling of S5 by GTP and nonspecific labeling of S4. The presence (+) or absence (-) of 10 μM GTP or ATP is indicated below the figure. GTP blocked photoinduced radiolabeling of S5 while ATP blocked radiolabeling of S4.

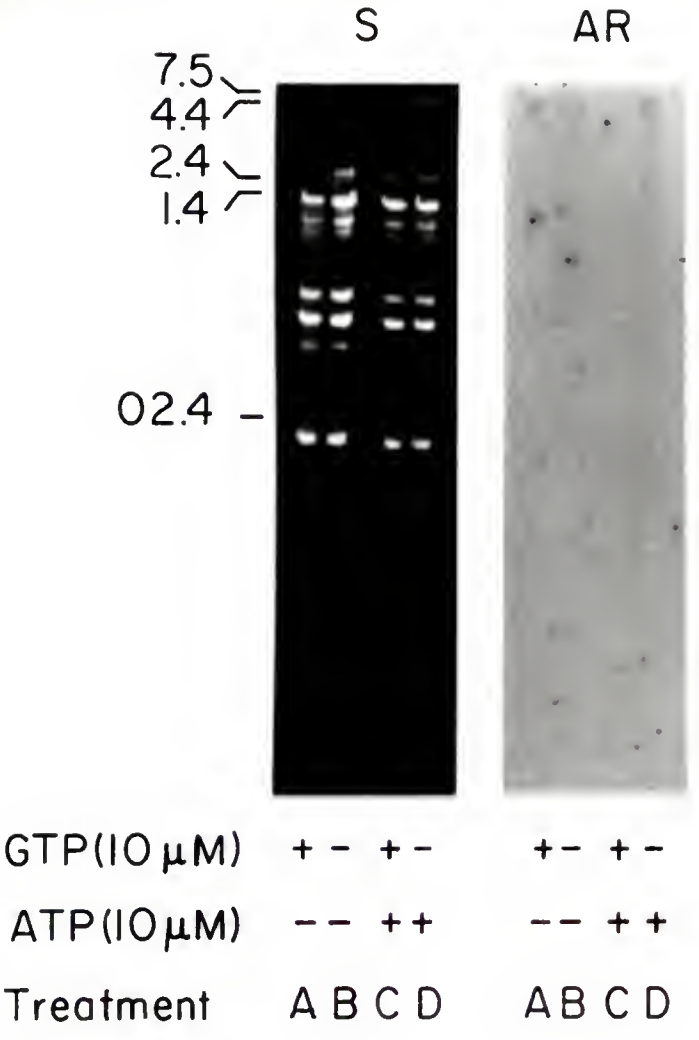


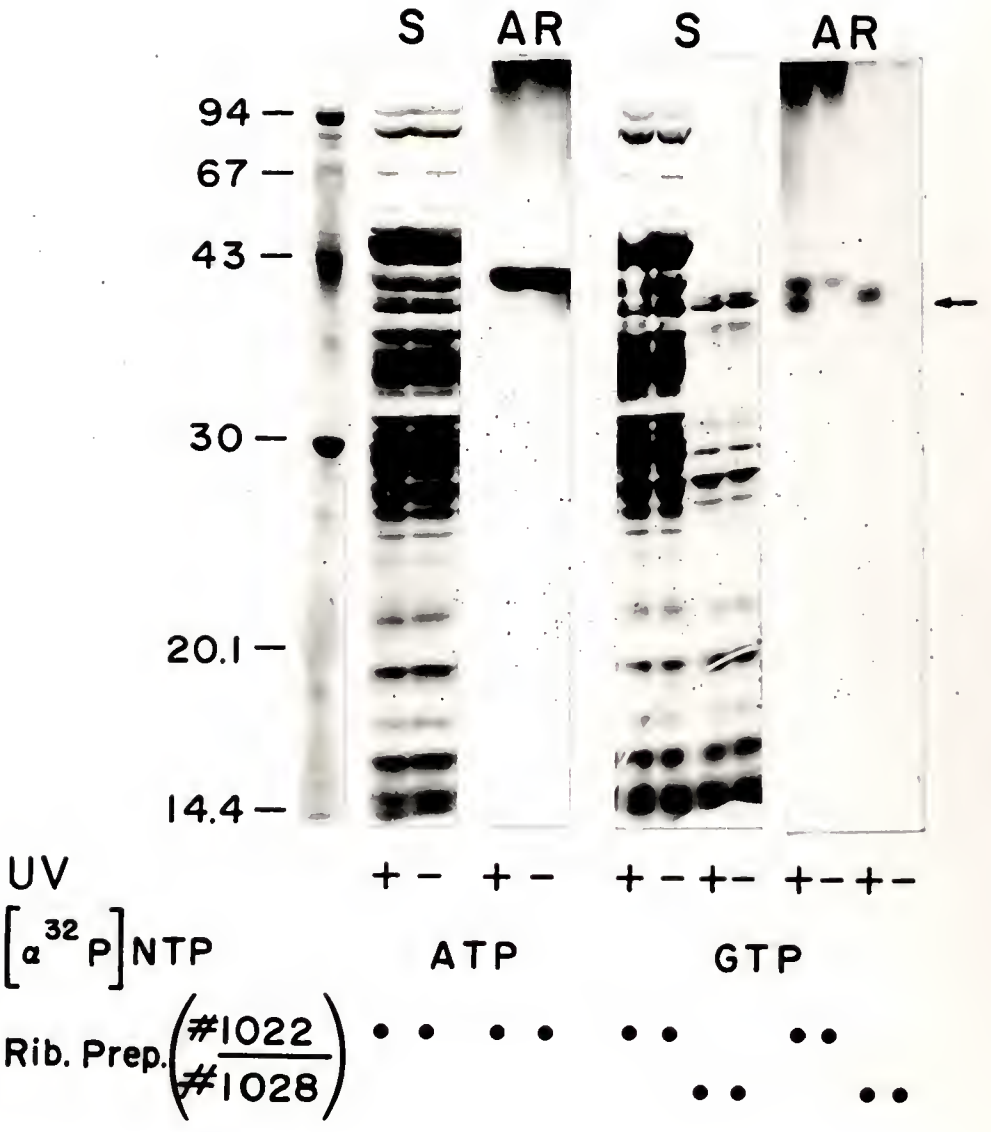
Figure 4-4 F Autoradiographic analysis of rRNA extracted from 28S ribosomes irradiated in the presence of [α^{32}P]GTP. In panel F, an analysis of the rRNA extracted from photoaffinity labeled 28S ribosomes shown in panels A to D. Following uv irradiation the rRNA was extracted in 9M urea and 3M LiCl and electrophoresed by 1D urea PAGE as described in Methods. The left panel is a methylene blue stained gel (S) and its corresponding autoradiogram (AR) on the right. The autoradiogram was exposed for 30 days. The molecular weight markers are six synthetic poly(A)-tailed RNAs ranging in length from 0.24 to 9.49 kb (BRL). No rRNA fragments were radiolabeled.

[α^{32}P]GTP in reaction buffer A as described above. In the absence of 10 μM GTP, S5 was specifically radiolabeled with [α^{32}P]GTP (figure 4-4) as was the case for [α^{32}P]8-Azido GTP (figure 3-3) and [γ^{32}P]8-Azido GTP (figure 3-7) but the majority of the radiolabeling was still on the nonspecifically labeled ribosomal protein, S4 as seen before. However in the presence of 10 μM ATP, radiolabeling of S4 was blocked completely, and the specific radiolabeling of S5 was more clearly visualized (figure 4-4 C and D). Radiolabeling of S5 by [α^{32}P]GTP was completely blocked in the presence of 10 μM GTP (figure 4-4 B, D and E) as seen in all previous studies with [α^{32}P] and [γ^{32}P]8-Azido GTP. This suggests strongly that the site of the specific and high affinity GTP binding activity observed on the small subunit of the mitochondrial ribosome is on ribosomal protein, S5. Autoradiographic analysis of 12S rRNA suggested no photolabeling occurred (figure 4-4 F)

Continued Radiolabeling Studies on S4

In an earlier experiment an interesting observation was made. In the absence of uv irradiation [α^{32}P]ATP radiolabeled the 43 kDa (S4) ribosomal protein (data not shown) for a particular preparation of 28S ribosomes. In figure 4-5 radiolabeling of 28S ribosomes in the presence and absence of uv (254 nm) irradiation was investigated. Two different preparations of 28S ribosomes (0.5 μM) were incubated with

Figure 4-5 A Determination of the requirement for uv irradiation to radiolabel 28S ribosomes with [α^{32}P]ATP and [α^{32}P]GTP. Duplicate samples of 28S ribosomes (0.5 μM) were incubated in reaction buffer A with either 0.26 μM [α^{32}P]ATP or [α^{32}P]GTP (50% saturation, based on an apparent Kd of 15.2 nM). One sample was irradiated with uv light for 10 min with 800 μW/cm^2 on ice as described in Methods and the other incubated on ice under dim light with no irradiation. One ribosomal preparation (#1022) was used for both NTPs and a second preparation (#1028) for [α^{32}P]GTP only. The silver stained (S) gels are on the left of their corresponding autoradiograms (AR). The molecular weight markers are the same shown in figures 3-2. The irradiation with uv light, [α^{32}P]NTP, and ribosome preparation used are indicated below figure. The specific activity of both [α^{32}P]NTPs was 122 Ci/mmol. The autoradiograms were exposed for 4 days. The arrow denotes the location of S5 (38 kDa).


S
AR
S
AR
94
67
43
30
20.1
14.4
UV
+ − + − + − + − + −
[α 32 P]NTP
ATP
GTP
Rib. Prep. (#1022 / #1028)

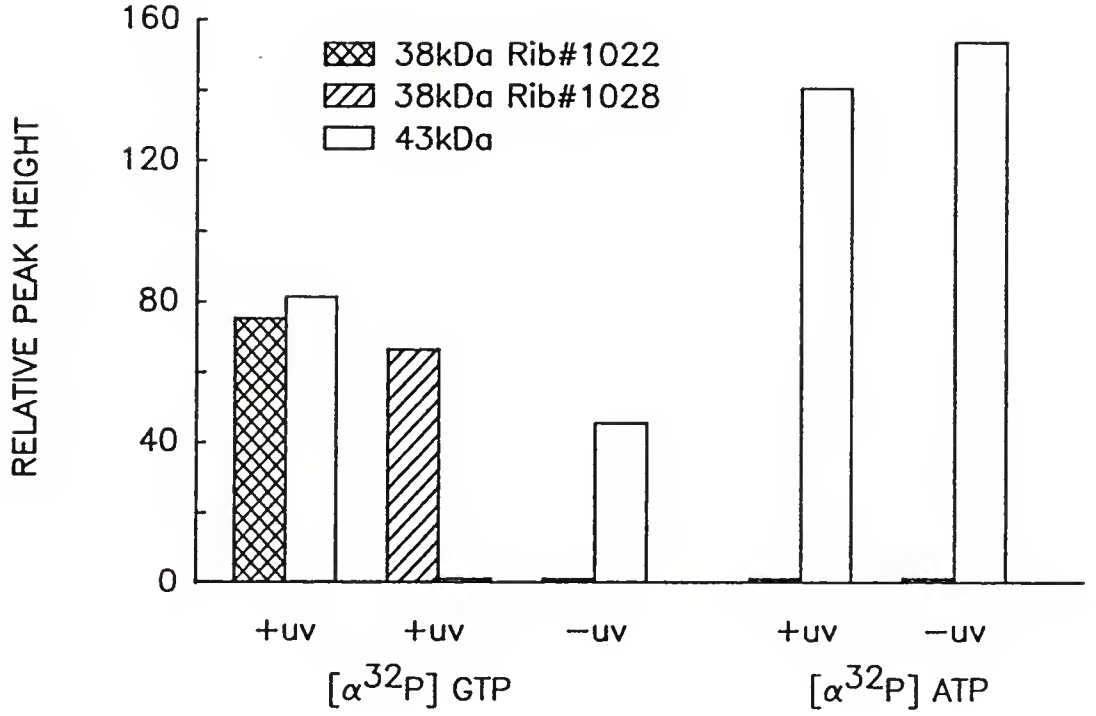


Figure 4-5 B Densitometric analysis of 28S ribosomes incubated in the presence of [α^{32}P]ATP and [α^{32}P]GTP in the presence (+) and absence (-) of uv irradiation. The autoradiogram shown in panel A was analyzed by densitometry and shown in panel B. The irradiation of the samples with uv light, [α^{32}P]NTP, and ribosome preparation used is indicated below the figure. For additional experimental details refer to Methods.

either 0.265 μM [α^{32}P]GTP or [α^{32}P]ATP in reaction buffer A and half the samples were irradiated with 800 μW/cm^2 uv light for 10 min on ice and under dim room light. The concentration of [α^{32}P]GTP used corresponds to 50% binding (based on a Kd of 15.2 nM for GTP) of the GTP binding site on the ribosome.

Ribosomal preparation #1022, S4 was radiolabeled by both [α^{32}P]GTP and [α^{32}P]ATP even in the absence of uv irradiation (figure 4-5 A, see AR lanes for rib. prep. #1022). In the other ribosomal preparation (#1028) radiolabeling of S4 was not detected (fig 4-5 A, see last two AR lanes on far right of figure) . Silver staining of the two ribosomal preparations resolved on a Laemmli gel indicated that S4 was absent in second preparation (#1028) likely accounting for this discrepancy (figure 4-5 A, S lanes). Ribosomal protein S5 was not radiolabeled by [α^{32}P]GTP or [α^{32}P]ATP in either ribosomal preparation in the absence of uv irradiation. S5 was only radiolabeled by [α^{32}P]GTP in the presence of uv irradiation (figure 4-5 A and B). The radiolabeling of S4 in the absence of uv irradiation not only survived boiling for 4 min in Laemmli sample buffer which contained 2% SDS but also resolution on a reducing Laemmli SDS polyacrylamide gel. Clearly radiolabeling of S4 by [α^{32}P]GTP or ATP occurred by a different mechanism than the photolabeling of S5 by [α^{32}P]GTP.

To determine if the uv independent radiolabeling of S4 by [α^{32}P]ATP has properties of a enzymatic reaction suggesting a catalytic site on the ribosome a time course (figure 4-6) for

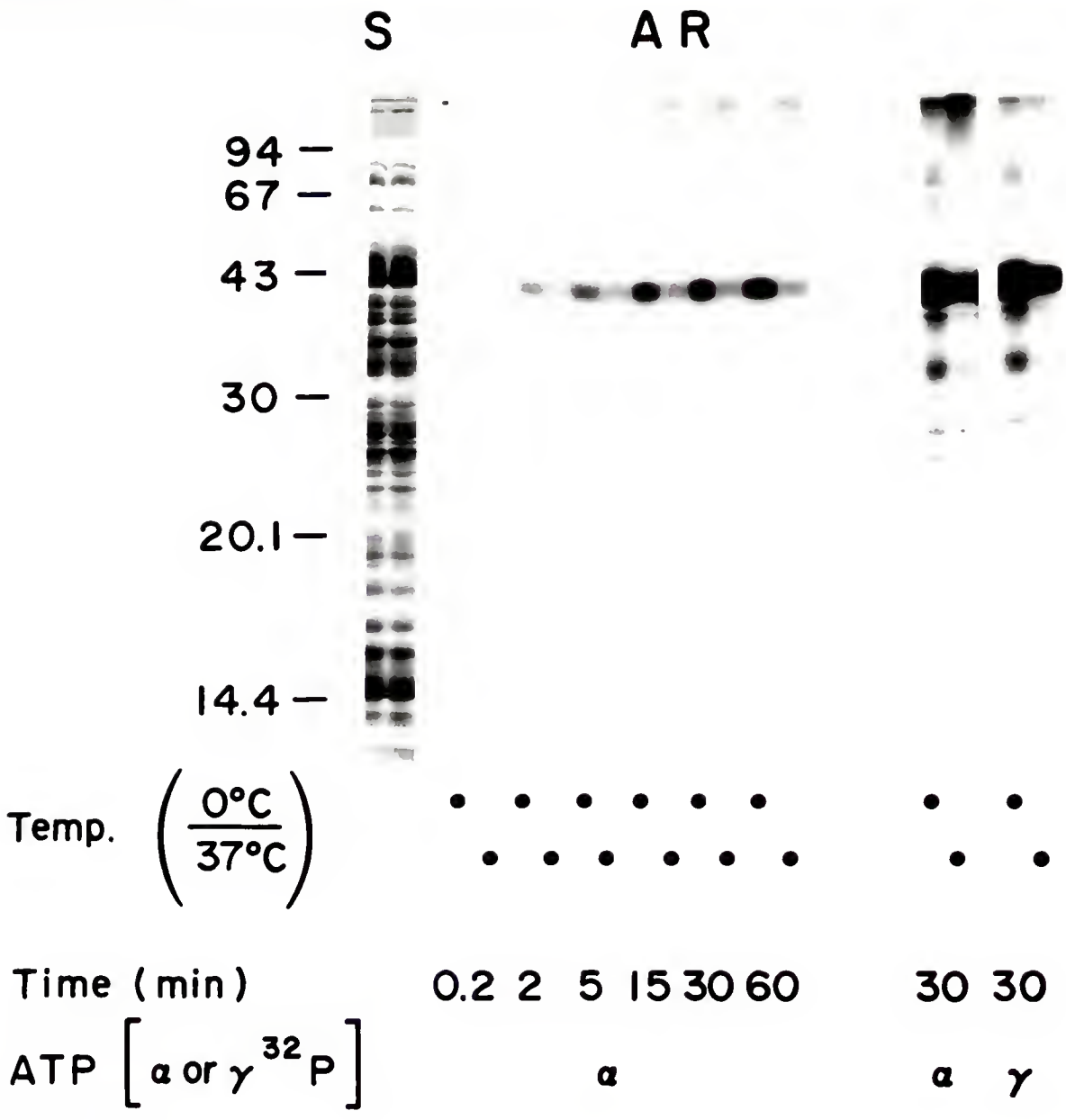


Figure 4-6 A Time course for the uv independent radiolabeling of S4 with [α^{32}P] and [γ^{32}P]ATP. Duplicate samples of 28S ribosomes (0.5 μM) were incubated in reaction buffer A with 1.0 μM [α^{32}P] for varying times (0.2 to 60 min) and 1.0 μM [γ^{32}P]ATP for 30 min. One of the duplicate samples was incubated on ice (0°C) and the other at 37°C under dim light. In panel A a representative silver stained (S) gel is shown on the left and the corresponding autoradiogram (AR) on the right. On the far right a AR comparing radiolabeling of ribosomes with [α^{32}P] and [γ^{32}P]ATP is shown. The arrow indicates the presence of S4 protein. The [α^{32}P] and [γ^{32}P]ATP were diluted 10 and 100 fold with ATP to a specific activity of 30 and approximately 70 Ci/mmol respectively. The arrow identifies S4 protein (43 kDa). The autoradiogram was exposed for 45 min.

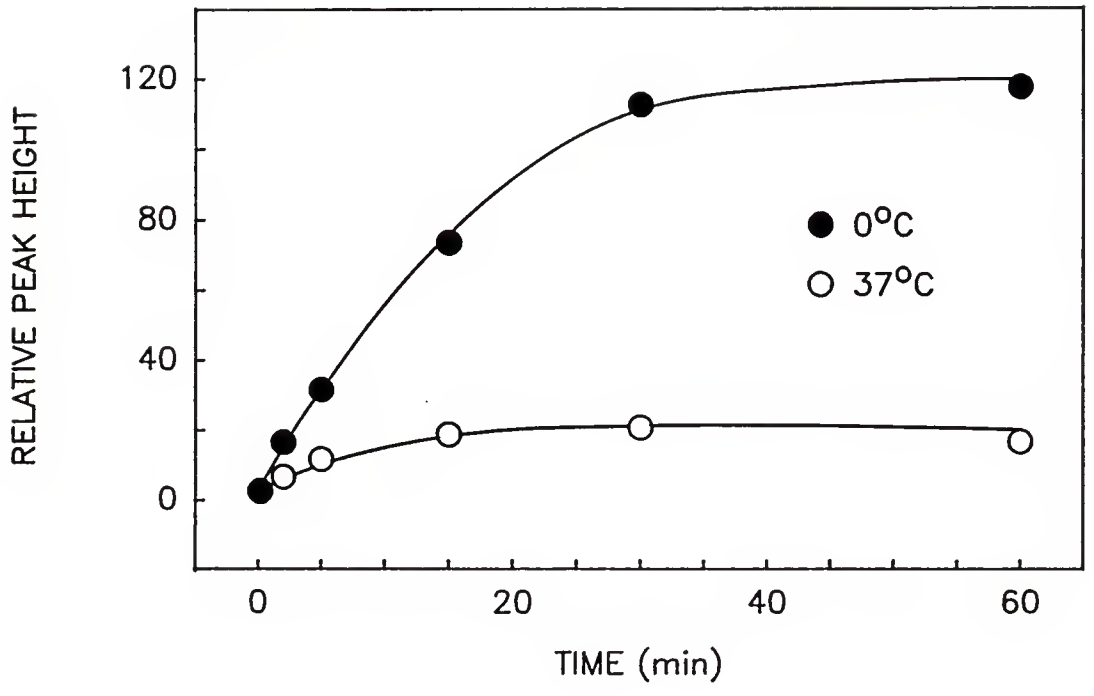


Figure 4-6 B Densitometric analysis for the radiolabeling of S4 by [α^{32}P]ATP at 0°C and 37°C over increasing length of time. Densitometric analysis was performed on the autoradiogram in figure 4-6 A. Refer to Methods for additional experimental details.

the radiolabeling of 28S ribosomes by [α^{32}P]ATP was done at 37° and 0°C. Small subunit ribosomes (0.5μM) were incubated with either 1μM [α^{32}P] or [γ^{32}P]ATP diluted with unlabeled ATP reaction buffer A for an increasing period of time at 0°C or 37°C. Radiolabeling did increase with time at 0°C leveling off after 30 min but almost no labeling occurred at physiological temperature (figure 4-6 A and B). This suggested that the radiolabeling of S4 by [α^{32}P]ATP was not enzymatic in nature. Additionally, comparison of the radiolabeling patterns (figure 4-6 A right hand panel) of [α^{32}P]ATP and [γ^{32}P]ATP were identical thereby not revealing any autophosphorylation of 28S ribosomes by [γ^{32}P]ATP.

To summarize the finding for the radiolabeling of S4 it was found that [α^{32}P]ATP failed to bind to 28S ribosomes in a comparable manner as did [α^{32}P]GTP as analyzed by filter binding assays (figure 2-1). However [α^{32}P]ATP did appear to bind to a fractional site on the ribosome (n=.09) with an apparent Kd of 77 nM (table 2-1). Both [α^{32}P]GTP and ATP radiolabel S4 independent of uv irradiation and that this activity was not found in all preparations of ribosomes (figure 4-5 A and B). ATP was the only nucleotide that could block radiolabeling of 28S ribosomes by both [α^{32}P]ATP and [α^{32}P]GTP (figures 4-2, and unpublished data). The radiolabeling of [α^{32}P]ATP occurs at 0°C and not at physiological temperatures and is time dependent (figure 4-6).

Discussion

The natural ligand GTP and its photoreactive analog, 8-Azido GTP bind specifically to S5. Binding to this protein was revealed by autoradiographic analysis of one and two-dimensional polyacrylamide gels following photoaffinity labeling of 28S ribosomes. The photolabeling of S5 by [α^{32}P]GTP could be blocked effectively only with GTP and GDP which agrees not only with the results of filter binding inhibition assays (figure 2-2) but also with photolabeling experiments using radiolabeled 8-Azido GTP (figure 3-3 and 3-7).

Following photoaffinity labeling a very small percentage (< 0.1%) of GTP or 8-Azido GTP remains covalently bound to the ribosomal protein. The addition of three negative charges (from the triphosphate group of the radioligand) to an average protein of 40 kDa mass is not expected to have much of an effect on the electrophoretic mobility of the protein in the two dimensional electrophoretic system used in these experiments (Matthews, et al., 1982). Superimposition of the autoradiogram with the dried two-dimensional gel revealed the radiolabeled protein to be S5. Most of the individual ribosomal proteins have distinctive shapes, which aids in the identification of radiolabeled proteins, especially if the mobility of the labeled protein differs somewhat from that of the unlabeled protein. The radiolabeled image of S5 matched precisely the corresponding silver stained protein spot on the two-dimensional polyacrylamide gel (figures 3-3, 3-7, and

4-4). This suggests strongly that S5 with the covalently bound nucleoside triphosphate migrates in the same manner as S5. The additional three negative charges did not retard the migration of S5 in the first dimensional separation to any detectable degree, since the separation of proteins in this first dimension is not as sensitive to alterations in charge, as in isoelectric focusing. Moreover, other labeling studies with mammalian mitochondrial ribosomes, such as iodination (Denslow and O'Brien, 1984) and affinity labeling with chloramphenicol analogs (Harville and O'Brien, unpublished data), indicated the labeling did not affect the mobility of ribosomal proteins in this electrophoretic system (Matthews, et al., 1982).

Photolabeling of S5 was saturable and quantification of the covalently bound [α^{32}P]GTP revealed the radiolabeling reaction to have the same concentration dependence as GTP binding to ribosome subunits determined in the filter binding assays (figure 4-3). This suggested that specific binding of GTP to the 28S ribosome was due to binding to S5.

The nonspecific labeling of S4 was independent of uv irradiation. It survived not only strong denaturing conditions, such as boiling in SDS and migration in SDS containing polyacrylamide gels, but also extraction in 10 M urea and 3 M LiCl for 2-dimensional PAGE analysis. Radiolabeling of S5 requires uv irradiation suggesting strongly these two proteins bind GTP by different mechanisms.

Radiolabeling of S4 also had a curious pattern. In the presence of excess amounts of GTP, which would block radiolabeling of S5, radiolabeling of S4 would not only not be blocked, but would increase in an apparently reciprocal manner. It was apparent that some of the radio-labeled GTP or even its 8-Azido analog that was displaced by GTP now bound to S4. The increase in radiolabeling of S4 even when the specific activity [α^{32}P] was diluted, by the addition of excess amounts of GTP, was unexpected. One explanation would be that S4 has a low affinity for GTP. Protein S4 does not bind GTP specifically and binds [α^{32}P]ATP as well. In fact ATP is the only nucleotide found that blocks radiolabeling of S4 by [α^{32}P]GTP or [α^{32}P]ATP. This finding was used in 2-dimensional analysis of photoaffinity labeling of 28S ribosomes by [α^{32}P]GTP to block labeling on S4 to more clearly demonstrate specific labeling of S5 (figure 4-4).

Scatchard analysis of GTP binding to ribosomes by filter binding assays revealed only one high affinity binding site for GTP on the ribosome with no indication that another low affinity site existed. This is likely due to the fact that these assays cannot measure binding with dissociation constants in the high μM range or higher. This is due to washing steps, used in filter binding assays to separate bound from free ligand. Ligands that bind loosely have higher off rates thereby are more susceptible to loses during washing. An alternate method that may reveal a putative second lower

affinity site would be equilibrium dialysis however we lacked sufficient quantities of ribosomes required for this analysis.

However quantitative analysis of the amount of [α^{32}P]GTP covalently bound to S5 and S4 in photolabeling experiments, was done and the shape of the binding curves do reveal differences in binding affinity (figure 4-3 B). The curve for S5 was very steep and hyperbolic. Scatchard analysis of the covalently bound [α^{32}P]GTP revealed a high affinity binding activity equivalent to that seen in filter binding assays. This suggested that S5 was the high affinity binding site for GTP on the ribosome. This calculation was based upon the assumption that at saturation, one molecule of GTP was bound to the ribosome as determined by filter binding assays. The binding curve for GTP to S4 (figure 4-3 B) was nearly linear and did not increase as rapidly as for S5 suggesting a low affinity by [α^{32}P]GTP for this protein. A dissociation constant could not be calculated because saturation was not demonstrated and the number of binding sites for GTP to S4 was not known.

The nature of the binding of S4 to ATP or GTP is also unknown. Since irradiation by uv was not required (figure 4-5) the binding occurs by a different mechanism than seen for S5. The binding to S4 was resistant to moderate salt concentration and was enhanced at low temperatures (0°C) versus physiological (37°) temperatures suggesting the binding is more hydrophobic in nature than electrostatic. The enhance-

ment in binding at low temperatures also suggests no enzymatic action such as seen in ADP ribosylation. Ribosylation of the ribosome can be ruled out not only because of the low temperature but because the label resided in the $\alpha^{32}P$ and not in the ribose and no NADP was added to the reaction. Additionally phosphorylation was ruled out because [$\alpha^{32}P$]GTP was used so no label resided on the γ-phosphate. Additionally, when [$\gamma^{32}P$]GTP was used no differences were seen in radiolabeling patterns between the two isotopes (figure 4-6).

The uv independent binding was able to survive harsh denaturing conditions such as boiling in SDS and migration in SDS containing polyacrylamide gels. The reason for such binding behavior is not readily apparent.

CHAPTER 5

ISOLATION, IDENTIFICATION, AND PARTIAL AMINO ACID SEQUENCE OF S5 AND OTHER RIBOSOMAL PROTEINS

Introduction

Separating ribosomal proteins from any source has always been a formidable task due to their similarities in molecular mass (6 to 60 kDa), large number of proteins in the various subunits (especially in the mitoribosome, see Table 1-1), isoelectric point (8 to 12), and in some cases extreme hydrophobicity (Kaltschmidt, 1971; and Matthews et al., 1982). The classical methods, ion exchange and size exclusion chromatography are quite laborious, provided inadequate resolution of individual proteins and suffered from low recovery of protein. Recently reverse phase high performance liquid chromatography (RP-HPLC) has become the method of choice. Kerlavage et al.(1983) resolved nearly all of the ribosomal proteins from all subunits in E. coli. Hirano et al. (1987) also separated ribosomal proteins from B. stearothermophilus and obtained amino-terminal sequence information from several of the proteins. These separations were done on a wide pore reverse phase alkylsilane columns of varying carbon chain lengths, with an increasing gradient of organic solvent in the presence of triflouroacetic acid (TFA).

The majority of these bacterial proteins separated as pure proteins. Three properties of this separation method that were most responsible for the high degree of resolution were: the requirement for a modifier, TFA, presumably to act as a counter-ion; the advent of wide pore (300Å) silica beads to allow passage of larger molecules; and the high resolving capabilities of HPLC columns with theoretical plate numbers of >10,000. The use of RP-HPLC with organic solvents, acetonitrile or 2-propanol, and TFA as a modifier has gained widespread use in separating not only ribosomal proteins but other proteins as well (Kamp et al.,1984; and Kamp, 1986).

A variation of the above method has proven useful when one deals limiting amounts of protein, narrowbore RP-HPLC. Narrow bore RP-HPLC was used extensively in this study to isolate ribosomal proteins to purity in order to conserve ribosomal protein as well as other reagents. The method used is described below in Methods, and simply utilized a column with a reduced inner diameter (2.1 mm). Narrow bore columns are operated at linear flow velocities equivalent to conventional bore columns (4.6 mm, I.D.) columns by decreasing the flow rate to the square of the reduction in column diameter as shown in the equation:

$$\text{Flow a} = (r_a / r_b)^2 \text{ (Flow b)}$$

For example, an equivalent flow rate for a narrow bore (r_b) in comparison to a conventional (r_a) HPLC chromatographic separation at a flow rate of 1 ml/min would be 0.047 ml/min.

Also assuming the column efficiency is nearly the same and the column capacity is not exceeded the same amount of protein would be eluted into a smaller volume of solvent thereby increasing the sensitivity by about 7 to 10 fold. The conservation of protein and smaller volumes was an asset in obtaining amino acid sequence information by micro gas-phase sequencing methods employed in this study.

In some cases ribosomal proteins were not obtained in sufficient purity in the RP-HPLC eluant or for other reasons were digested into peptides. These were further resolved on one-dimensional Laemmli gels and electroblotted onto a solid phase support for sequence analysis. Western blotting of proteins from PAGE gels onto TFA derivatized glass fiber sheets was initially described by Aebersold et al. (1986) and Vandekerckhove et al.(1985). However with the use of glass fiber supports initial and repetitive cycle yields often decreased with decreasing amounts of protein and small quantities (<100 pmol) proved difficult to sequence (Yuen et al., 1986). The search for a superior blotting support has led to the adoption of PVDF which has proven especially useful for electroblotting of hydrophobic proteins and peptides (Pluskal et al., 1986, and Matsudaira, 1987).

I report here the separation of 28S ribosomal proteins and the ribosomal identity of most of the eluted proteins. Most of the identities were determined by coelectrophoresis of eluted proteins with a small amount of 28S proteins on

two-dimensional gels. I also will show S5, the GTP-binding protein, elutes as a pure protein and identified not only by the above method but also by immunoblot analysis with a anti S5 monospecific antibody. The amino-terminal sequence of a number of 28S ribosomal proteins as well as considerable amino acid sequence derived from peptides of S5 will also be shown. Homologies in the amino acid sequence found in peptides of S5 to conserved regions in other GTP-binding regions involving not only the triphosphate chain but also the guanine base will be discussed.

Materials and Methods

RP-HPLC Mobile Phase Solutions

Aqueous Mobile Phase: HPLC grade water (Fisher Scientific) with 0.1% trifluoroacetic acid (TFA, sequencer grade)

Organic Mobile Phase: HPLC grade 2-propanol (Fisher Scientific) with 0.1% TFA

Electroblotting Solutions

Blotting Buffer: 0.25 M Tris-HCl, pH 8.3, 20% HPLC grade methanol (Fisher Scientific), 192 mM glycine, and 0.01% SDS (ultrapure grade)

Destain Solution: 50% HPLC grade methanol, and 2% HPLC grade acetic acid

Immunoblot Solutions

Blocking Solution: 3% Bovine Serum (v/v), 150 mM NaCl, 10mM Tris-HCl, pH 7.5, and 0.01% sodium azide

Incubation Buffer: 3% Bovine Serum (v/v), 150 mM NaCl, 10 mM Tris-HCl, pH 7.5, 0.01% sodium azide, 0.5% Triton X-100, and 0.01% SDS

TBS: 10 mM Tris-HCl, pH 7.5, and 150 mM NaCl

TBSA: 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.01% Na-azide

Column Elution Buffer: 0.5 M HPLC grade acetic acid, and 1% dioxane

Equipment

1) High Pressure Liquid Chromatograph: Hewlett Packard model #1090A with integral photo-diode array detector. The detector has a Hewlett Packard model# 9121A double 3.5 in disk drive, model# 65 personal computer, and a model# 7470 plotter device. The diode array detector was capable of storing up to 8 distinct wavelengths simultaneously and collecting spectral information as desired. One signal was split (signal 1, at 214 nm) to be analyzed by a Hewlett Packard model# 3390A integrator. The chromatogram had a built in column oven to maintain a constant solvent and column temperature.

2) Gas Phase Amino Acid Microsequencer: Applied Biosystems (ABI, Foster City, CA) model# 470A with on-line ABI model# 120A PTH amino acid analyzer. All solvents used in conjunction with this instrument were purchased from Applied Biosystems Incorporated and were of Sequencer grade.

3) Savant Speed Vac concentrator, model# SVC-100H

4) IEC lab top general purpose centrifuge

5) Beckman table top high speed microcentrifuge

6) 96 well dot blot apparatus

7) Electroblotting device, Bio-Rad model# transblot

Extraction of Ribosomal Proteins for RP-HPLC analysis

Ribosomes were extracted in $Mg(Ac)_2$-glacial acetic acid to remove rRNA by a modified procedure of Hardy et al. (1969) prior to RP-HPLC analysis. To 28S or 39S ribosomes 1 M $Mg(Ac)_2$ was added to a final concentration of 32.2 mM and allowed to stir for 5 min at 4°C. Concentrated HPLC grade glacial acetic acid was then added to a final concentration of 66% of the total volume. The mixture was stirred at 4°C for 1 h, then centrifuged at 140,000 x g for 30 min at 4°C. The rRNA pellet was re-extracted as described above for 20 min and the supernatants pooled and dialyzed against 6% HPLC grade acetic acid for 2 h prior to RP-HPLC analysis.

Reverse Phase HPLC of Ribosomal Proteins

Purification of mitochondrial ribosomal proteins for chemical microsequencing purposes was accomplished by RP-HPLC. Chromatographic separations were accomplished with a Hewlett Packard 1090A HPLC with a photodiode array detector. This detector allowed multiple wavelengths to be monitored during an analysis. This capability allowed for the subtraction of a reference signal of a wavelength (540 nm) from the signal of interest (219, and 280 nm) which resulted in a chromatogram largely devoid of baseline drift due to changes in solvent composition during gradient runs. Spectral information for all peaks were also stored at a 4 nm intervals between 210 and

450 nm for subsequent analysis if required. Data acquired was stored on 3.5 in floppy disks and data analysis performed on an integral Hewlett Packard personal computer (model #65).

Chromatographic separations were achieved on a wide pore silica based butyl reverse phase columns purchased from Synchrom. These columns have a bead size of 7 microns and a pore diameters of 300 Å which allow for the for the separation of proteins to a maximum M_r of 300 kDa. Two sizes of this type column were employed, a 4.1 X 250 mm column or a narrowbore column of 2.1 X 250 mm I.D. The conventional column (4.1 X 250 mm) was used for those experiments where larger amounts of protein, usually >400 pmol,were needed. Such was the case in those experiments where the identity of the proteins purified by RP-HPLC was determined by coelectrophoresis of eluted peaks on 2D-PAGE. The narrowbore column was primarily used when <400 pmol of total protein was needed as was the case for microsequencing of purified proteins. The narrowbore column operated at approximately one third the flow rate and at least four times the sensitivity of the conventional column (Simpson and Nice, 1987), which allowed for the analysis of 100 pmol of ribosomal protein. Both columns used the same bonded phase butyl packing material and nearly identical separations resulted.

Mitochondrial ribosomal proteins were resolved on the silica-C4 HPLC column by a gradient elution of increasing proportions of 2-propanol versus water over a 10 h time

period. Both the aqueous and organic solvents contain 0.1% TFA, as a counter ion to charged amino groups. Flow rates were 250 µl/min from 0 to 260 min and then reduced to 200 µl/min until the end of the analysis for the conventional column. For the narrowbore RP-HPLC column, a flow rate of 200 µl/min was maintained for the first 100 min while the samples were loaded onto the column in multiple injections at 1% organic solvent. During gradient elution the flow rates were 80 µl/min from 100 to 260 min and then reduced to 64 µl/min until the end of the analysis. The gradient profile for both columns was as follows: 0 to 100 min, 1% organic; to 120 min, 12%; to 140 min, 19%; to 155 min, 22%; to 260 min, 28%; to 570 min, 42%; to 600 min, 55%; to 630 min, 75%; and to 635 min, 25% 2-propanol. The column was maintained at a constant temperature of 35°C during an analysis. Samples were injected at 1% 2-propanol by multiple injections until all the samples had been injected. This was possible because proteins exhibit high binding capacities below critical organic solvent composition on reverse phase supports. Therefore it was possible to concentrate large sample volumes directly on the column before recovery of proteins by gradient elution (Nice et al., 1979). Samples were collected into 1.5 ml plastic screw cap tubes at 1 to 5 min intervals with the aid of a fraction collector.

Gas Phase Protein Sequence Analysis:

Determination of primary sequence of ribosomal proteins was done by Edman (1956) degradation. Edman degradation is a repetitive reaction in which the alpha amino group of a polypeptide chain is chemically coupled to phenylisothiocyanate (PITC) under alkaline conditions with trimethylamine (TMA). The amino terminal derivative is then cleaved in the presence of trifluoroacetic acid (TFA). resulting in an anilinothiazolinone (ATZ) derivative. This derivative is then converted to a stable phenylthiohydantoin (PTH) amino acid in the presence of TFA and water, which was then analyzed by RP-HPLC.

Polypeptide sequence analysis was performed with a 470 gas phase protein sequenator with a 120A on line PTH-HPLC analyzer (Applied Biosystems). Samples sequenced were bound to polyvinylidene difluride (PVDF) membrane by electroblotting proteins from 1D-SDS gels.

Proteins or peptides were sequenced and the resulting PTH-amino acids were analyzed on a model 120A PTH-HPLC analyzer using programs 03RPTH. This chromatographic program consisted of a 2.1 X 220 mm C18, HPLC column (model PTH C18, ABI, Foster City, CA). The bead size of the C18 column packing was 5 microns. The aqueous solvent was 60 mM sodium acetate, pH 4, and the organic solvent was acetonitrile with 3.5% tetrahydrofuran. The flow rate was 200 µl/min and separation of PTH residues was achieved by an increasing

gradient of organic solvent. The gradient profile for this HPLC method was as follows: 0 to 9 min, 8%; to 11 min, 15%; to 11.3 min, 16%; to 29 min, 36%; to 40 min, 36%; to 40.1 min, 80%; and continued at 80% until the end of the chromatogram at 47 min. Repetitive yields were determined by comparison of peak heights of Val, Leu, Glu, to peak heights of these same residues as they occur later in the sequence. The decrease in yield of these residues versus the number of cycles approximated the repetitive yield.

CNBR Digestion of Ribosomal Protein

To obtain internal amino acid sequence of a ribosomal protein the protein was chemically digested with CNBR. In 70% formic acid CNBR cleaves polypeptides on the carboxyl side of methionyl residues (Gross and Witkop, 1961). The CNBR peptides were resolved on a 15% polyacrylamide Laemmli gel then electroblotted onto PVDF membrane for gas phase microsequencing. This method was applied to one ribosomal protein, S5, for which amino-terminal sequence could not be obtained, presumably due to chemical blockage of the amino terminus. The protein was first isolated in pure form by narrow bore RP-HPLC and the fractions containing the pure protein pooled and concentrated to near dryness (approximately a 10 µl volume) in a speed vac centrifuge. The sample was brought to a 20 µl volume with water and then 88% formic acid (ACS grade, Fisher Scientific) was added to a final concentration of 70% formic acid with a resulting volume of

100 µl. Approximately 1 mg of CNBR powder was quickly added to the sample under a fume hood. After the CNBR was in solution a stream of N_2 gas was passed over the sample for 15 sec and the tube was capped quickly. The sample was wrapped in foil to exclude light and allowed to digest at room temperature for 16 h.

To resolve the CNBR peptides the sample was prepared for gel electrophoresis. Laemmli sample buffer (30 µl), with no bromophenol blue dye, was added to the sample and concentrated to near dryness in a speed vac centrifuge. Once the volume was approximately 30 µl, HPLC grade water was added to a final volume of 100 µl and speed vac concentrated again to near dryness. This step was repeated once more to reduce the concentration of the formic acid. A small volume (10 µl) of Laemmli sample buffer, with dye added, was again added to the sample and 5% TEA (triethanolamine) was added to the sample (approximately 60 µl) until the pH indicator dye turned blue to indicate the sample was slightly basic and then heated at 100°C for 4 min prior to electrophoresis. The CNBR peptides were resolved on a 15% polyacrylamide Laemmli gel and then electroblotted onto PVDF membrane as described below for amino acid sequence analysis.

Electroblotting of Ribosomal Proteins onto PVDF

Ribosomal proteins or CNBR peptides of ribosomal proteins were resolved on one dimensional SDS Laemmli gels and electroblotted onto PVDF membrane for amino acid sequence

analysis. Following electrophoresis, a wetted PVDF membrane was placed, in close contact, on one side of the 1D-SDS gel and then sandwiched between two sheets of wetted Whatman paper. The membrane was first wetted briefly in 100% methanol, then blotting buffer (0.025 M Tris-HCl, pH 8.3 , 20% HPLC grade methanol, 0.193 M glycine, and 0.01% SDS) for 10 min. Blotting was done in blotting buffer in an electroblotting apparatus (Bio-Rad, trans-blot model) at 60 V for 24 h at 4°C with the gel oriented so that the PVDF membrane was closest to the cathode. The electroblotted PVDF membrane was then lightly stained with Coommasie blue (0.1% in 50% methanol) stained for 5 min and destained in destain solution (50% methanol, and 2% acetic acid, both HPLC grade) for approximately 10 min until polypeptide bands were visible. Bands of interest were excised with a sterile scalpel from the membrane and placed in sterile vials and purged with nitrogen gas and stored at -20°C until analyzed.

Analysis of One and Two-Dimensional Immunoblots of 28S Ribosomal Proteins

Small subunit ribosomal proteins were resolved on either a 1-dimensional Laemmli gel (12% polyacrylamide) or 2-dimensional urea gel as described above. Small subunit proteins were electrophoresed on the Laemmli gel in a wide lane (5 cm) forming a curtain of resolved protein. Both types of gels were then electroblotted onto sheets of nitrocellulose or PVDF membrane. Electroblotting for either support was

identical as described above with the exception that the nitrocellulose need not be prewetted in 100% methanol. Following transfer onto the support, strips were cut (5 X 200 mm) parallel to the direction of electrophoresis to obtain resolved 28S ribosomal proteins for immunoblot analysis.

Electroblotting 2-dimensional gels did require equilibration of the gel in copious quantities of blotting buffer for 2 h before transfer could begin to remove excessive amounts of urea. Electroblotting of 2-dimensional gels was performed in the same manner as for Laemmli gels.

Immunoblot analysis for proteins electroblotted onto nitrocellulose or PVDF supports were done in an identical manner. Blots were first incubated for 30 min at 4°C in blocking solution (3% Bovine serum, 150 mM NaCl, 10 mM Tris-HCl, pH 7.5, and 0.01% sodium azide). Rabbit serum containing monospecific antibody against S5 was diluted 1:100 in incubation buffer (same composition as blocking solution with 0.5% Triton X-100, .01% SDS added) for analysis of 1D-Immunoblot strips. Anti S5 rabbit serum was depleted of antibodies against bovine liver proteins and E. coli lysates, as described below, and also diluted to a final ratio of 1:100 with incubation buffer prior to 2-dimensional immunoblot analysis.

Individual blots were incubated with the appropriate diluted anti S5 serum overnight at 4°C with gentle shaking. 28S Ribosomal strips and 2-dimensional blots were incubated in

approximately 1.5 and 15 ml of diluted serum, respectively. Blots were then washed for 20 min, 3 times in cold incubation buffer. A second anti-rabbit IgG (Fc) antibody alkaline phosphatase conjugate, was diluted 1:7500 with incubation buffer and incubated with the blots for 1 h at room temperature with gentle shaking. Blots were washed twice at room temperature while shaking with TBSA (150 mM NaCl, 10 mM Tris-HCl, pH 7.5, and 0.01% sodium azide) and a final wash with TBS (same as TBSA but no sodium azide present). Immunoreactive proteins were visualized using a color reaction provided in a ProtoBlot Immunoscreening Kit (Cat# P3710, Promega, Madison WI). Alkaline phosphatase conjugated to the Fc portion of a anti-rabbit IgG catalyzed the dephosphorylation of the substrate, BCIP (5-bromo-4-chloro-3-indolyl phosphate), which in turn reacted with NBT (nitro blue tetrazolium) to form a purple color, indicating immunoreactivity by the primary antibody.

Depletion of Anti S5 Rabbit Serum of Bovine Liver and E. coli Lysate Cross Reacting Antibodies

Rabbit serum containing monospecific antibodies against mitochondrial ribosomal protein S5, were depleted of antibodies that would bind to epitopes in both Bovine liver and E. coli lysates. This was done to reduce background signals for 2-dimensional immunoblot analysis with this rabbit serum. This was accomplished by passage of the rabbit serum, diluted 1:10 in incubation buffer, through 2 columns each

containing either liver or E. coli lysates conjugated to Reacto-Gel (HW# 65F, Sigma).

Conjugation of the Bovine liver lysate was accomplished by first chopping 8 g of commercially purchased frozen beef liver in 20ml of ice cold 0.1 M sodium borate buffer, pH 8.5 with 2% SDS. The liver was finely homogenized for a few seconds on ice with a small Tekmar tissue homogenizer. The volume was adjusted to 40 ml with 0.1 M sodium borate buffer with 2% SDS. The liver homogenate was then heated to 100°C for 5 min and placed in a 50 ml conical tube and centrifuged for 10 min at 1800 rpm in a IEC table top centrifuge at room temperature. 20ml of the supernatant was removed, added to 15 ml of React-Gel and adjusted to a final volume of 50 ml with cold 0.1 M sodium borate buffer. The coupling reaction was allowed to proceed for 60 h while stirring at 4°C. A small tubular glass column (1.5 X 8 cm) was packed with the liver lysate coupled gel, washed 5 volumes of 1 M NaCl, then with an equal volume of TBS. The column wash was repeated once more and the column was sealed and stored in TBS at 15°C until used.

The preparation of the E. coli lysate column was identical to that described for the liver lysate column. Only the preparation of the lysate differed. One liter of E. coli Y1090 was grown overnight at 37°C in Luria Broth with 0.2% maltose, 10 mM $MgSO_4$, 10 µg/ml tetracycline, and 40 µg/ml ampicillin. The cells were collected by centrifugation at 800

rpm for 5 min and solubilized gently in 20ml of TBS at pH 8.0. Lysozyme (Sigma) was added to concentration of 5 mg/ml and incubated for 1.5 h at room temperature. Tween 20 was added to a final concentration of 1% and vortexed repeatedly. 20% SDS was added to a final concentration of 2% and mixed with a vortex mixer again and allowed to incubate overnight at 4°C without shaking. The mixture was placed in a 100°C water bath for 5 min, then centrifuged at 1800 rpm for 10 min to sediment cellular debris. An 8 ml aliquot of the supernatant was diluted to 35 ml with 0.1 M sodium borate buffer and added to 15 ml of the Reacto-Gel and the coupling reaction done as described above.

The rabbit serum containing the monospecific anti S5 antibody was diluted 1:10 with cold TBS. The diluted serum was first passed through the E. coli lysate column which was equilibrated with 2 volumes of TBS. All column manipulations were done at 15° C in a cold box and all solutions used were also kept at that temperature. The column eluant passed through a 5 mm flow cell and the detector (ISCO, model#UA-5) signal was monitored at 254 nm and recorded on a Fisher Recordall (model# 5000) strip chart recorder. Following equilibration, a baseline recording was achieved, the diluted serum was added to the open column and passed through the column by gravity alone. When the diluted serum passed into the column bed completely the column reservoir was filled with TBS. The antibody peak was collected until the signal

returned to the baseline value. The column was regenerated by passing 20ml column elution buffer (0.5 M HPLC acetic acid, and 1% Dioxane) followed by an equal volume of TBS through the column. A peak of bound antibody, approximately one tenth the volume of the free antibody peak, eluted with the elution buffer. The column was then quickly neutralized with 1 volume (20ml) of 2 M Tris and the pH of the eluant was checked until it was above pH 9. Two volumes of TBSA were then passed through the column and the column stored three fourths full of TBSA at 15°C for future use.

The free antibody peak collected was next passed through the Bovine liver lysate column in the same manner as described above. Following passage through both columns the free antibody peak was diluted to a final ratio of 1:100 with incubation buffer and E. coli lysate was added to the serum to a final concentration of 10% (v/v).

Results

RP-HPLC of 28S Ribosomal Proteins and their Identification by 2D-PAGE Analysis

Photoaffinity methods identified S5 as the mitochondrial ribosomal protein responsible for specifically binding GTP. Reverse phase HPLC methods were employed to isolate and purify this protein and other 28S ribosomal proteins. The first task was to identify which peak S5 corresponded to and to assess its purity. The second task was to isolate adequate amounts

of pure S5 for gas phase chemical microsequencing to obtain partial amino acid sequence information for this protein.

Small subunit ribosomes were extracted in 66% acetic acid as described in Methods to remove rRNA and injected onto a wide pore (300 Å) butyl (C4) reverse phase HPLC column (4.1 X 250 mm). The extracted ribosomes were loaded onto the column in a series of 12, 100 µl injections spaced 5 min apart until approximately 1600 pmol of ribosomal protein was applied to the column at 1% 2-propanol. The ribosomal proteins were eluted from the RP-HPLC column with a gradient of increasing concentration of 2-propanol as described in Methods. Eluted fractions were collected at 2.5 min intervals and 10% of each fraction was removed for 1D-PAGE analysis, and the remainder left uncapped overnight in a fume hood to allow for the evaporation of the 2-propanol from the samples. Thirty µl of 50% ultra pure glycerol was added to the samples and then evaporated in a Savant speed vac apparatus to approximately one fourth their original volume or about 200 to 150 µl. The samples were overlaid with a stream of N_2 gas and quickly capped and stored at 15°C in the dark until needed for 2D-PAGE analysis.

Reverse phase high pressure liquid chromatographic separation of mitochondrial 28S ribosomal proteins has been developed to the extent that the majority of the ribosomal proteins can now be resolved to purity (figure 5-1). Co-electrophoresis of eluted peaks from the RP-HPLC C4 column

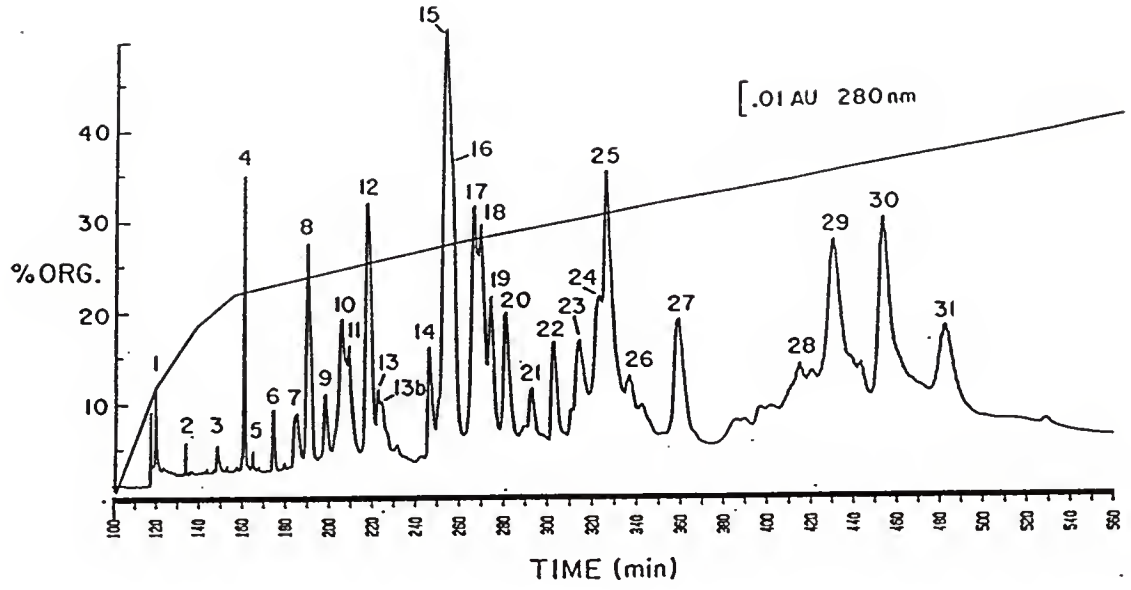


Figure 5-1 Reverse phase high pressure liquid chromatogram of 1.6 nmol of acetic acid extracted 28S ribosomes resolved on a wide pore (300 A) silica bonded butyl column. The ribosomal proteins were injected into the chromatograph in several small injection volumes (100 µl X 20) every 5 min at 1% 2-propanol (this part of the chromatograph is not shown). The proteins were then resolved with a increasing gradient of 2-propanol (%Org) versus water both with 0.1% TFA as described in Methods. Peaks were detected at 214 and 280 nm simultaneously and the 280 nm signal (at 0.1 AUFS) is shown in this figure. The eluted peaks are numbered sequentially as they elute from the column. The proteins comprising these peaks were identified by coelectrophoresis on 2D-PAGE gels with 28S ribosomes in the following figures (figure 5-2 A to Q). The peak numbering system used in this figure was used in this identification process. Fractions were collected at 2.5 min intervals starting at 110 min and continuing until 500 min or the end of the chromatogram. In table 5-1 the fractions and peaks selected for 2D-PAGE identification and their identities are summarized.

with 28S ribosomal proteins on 2D-PAGE gels has allowed for the identification of the proteins in 27 out of 31 peaks (table 5-1). S5 was a late eluting peak (#29) that contained no other proteins. The identity was determined by 2D-PAGE co-electrophoresis (figure 5-2 A to Q, and table 5-1) and immunoblot analysis (figure 5-5) with an anti S5 antibody that was confirmed to predominately recognize S5 by 2D-PAGE immunoblot analysis (figure 5-4).

Amino-terminal sequence has been obtained for four small subunit ribosomal proteins (table 5-2) isolated by RP-HPLC. Sequences were obtained in all cases with the use of PVDF membrane as the solid support for micro gas-phase sequence analysis. The technique for application of the samples to the support membrane varied in an effort to find a method that would yield optimal results (table 5-1). For those proteins eluted as pure peaks the samples could be either spotted directly onto the PVDF disks or allowed to bath in the peak eluant while shaking overnight. Both methods provided satisfactory results but samples must be allowed to stay in contact with the membrane for several hours to allow for complete absorption of the sample onto the membrane prior to sequencing. Another precaution that was necessary to prevent chemical blockage of the amino terminus by TFA present in the HPLC mobile phase was to not allow the proteins to remain in TFA for more than 48 h.

S5 was the only small subunit protein sequenced so far that apparently was naturally blocked at its amino-terminus preventing N-terminal sequence analysis. Amino-terminal sequencing of S5 was attempted twice and both times no sequence was obtained, even though analysis of the sample remaining on the PVDF membrane revealed at least 17 pmol (table 5-1) of protein as determined by acid hydrolysis followed by amino acid analysis on a Beckman 6300 AA analyzer (data not shown). The chemical nature of the amino-terminal blockage is unknown.

Identification of S5 and other ribosomal proteins eluted from the RP-HPLC column was accomplished by coelectrophoresis of eluted peaks with 28S ribosomal proteins on 2D-polyacrylamide gels. A 10 to 20 molar excess of HPLC resolved ribosomal proteins (figure 5-2) were added to a minimal amount of acetic acid extracted 28S ribosomes (30 pmol) and were dialyzed versus 2D-PAGE sample buffer overnight as described in Methods. Following 2D-PAGE resolution and silver staining of the gels, identification of the ribosomal proteins eluted by HPLC was made by comparison of the enhanced staining of those ribosomal proteins, present in 10 to 20 molar excess, to the background amounts of 28S ribosomal proteins. Additionally, a small amount (approximately 160 pmol) of the RP-HPLC resolved proteins were electrophoresed in a lane parallel to the second dimension to aid in identifying enhanced peaks. In figure 5-2 (A to Q) are 2D-PAGE gels with

the identity of the ribosomal protein present in the corresponding RP-HPLC peak. Table 5-1 summarizes the identity and purity of the proteins eluted from the RP-HPLC column. S5 was identified (figure 5-2 J) as peak #29, eluting as a pure peak at 36% 2-propanol or 428 min (figure 5-1).

Identification of S5 RP-HPLC Peak by Western Blot Analysis Using a Monospecific Antibody Raised Against S5

A monospecific antibody was raised against S5 and other small and large mitochondrial ribosomal proteins by Pietromonaco et al. (1986). Monospecific antibodies were prepared using proteins extracted from 2-dimensional gels bythe acetic acid method of Bernabeau et al. (1980). A rabbit was immunized with S5 extracted from gel plugs cut from five 2-dimensional gels. Each 2-dimensional gel was prepared with approximately 270 pmol of 28S ribosomal protein. This rabbit required 4 booster injections of S5 protein from the same number of gel plugs except, for the last injection where 10 gel plugs were used, before antibody was detected in the serum. The total period of time required for immunization of S5 in this rabbit was 6 months. Pietromonaco et al. (1986) reported a strong immunoreaction of a ribosomal protein on a 1-dimensional immunoblot corresponding to the Mr of S5.

Serum collected from this rabbit was stored at -20°C for approximately 5 years before use in this study. To ensure the quality of this sera immunoreactivity of 1-dimensional blots of 28S ribosomal proteins were examined. Frozen 350 µl

Figure 5-2 Two-dimensional PAGE analysis of 28S ribosomal proteins eluted from a RP-HPLC butyl column, coelectrophoresed with a minimal amount of acetic acid extracted 28S ribosomes. In gels B to J, 38 pmol of acetic acid extracted ribosomes were coelectrophoresed with approximately 5 to 20 molar excess of ribosomal proteins eluted from the butyl RP-HPLC column shown in figure 5-1 on a 1.5 X 160 X 200 mm slab gel. In gels L to Q 23 pmol of extracted ribosomes were used and coelectrophoresed with ribosomal proteins eluted from the butyl column as in the preceding gels. These 2D-PAGE gels were smaller in size, 1.5 X 80 X 100 mm. The same preparation of ribosomes was used for both RP-HPLC and 2D-PAGE coelectrophoresis. The proteins eluted from the RP-HPLC column and identified by 2D-PAGE were from a single chromatographic run shown in figure 5-1. In every 2D-PAGE gel a portion (approximately 5% v/v) of the eluted peak was run in a lane on the second dimension slab gel, parallel to the second dimension, to show the migration of the eluted protein(s) in the urea/SDS slab gel. Proteins isolated by RP-HPLC that were identified as an enhanced protein spot migrating approximately the same distance on the second dimension as the one dimensional lanes. These are identified by protein name on each figure. These fractions are labeled as to their peak (P, figure 5-1) and fraction (f) number. In table 5-1 the retention times and protein identities corresponding to these fractions from the RP-HPLC (figure 5-1) are summarized. Gels A and K are electrophoretic patterns of 28S ribosomal proteins, without any HPLC fraction added, electrophoresed under the same electrophoretic conditions for gel B to J and L to Q respectively.

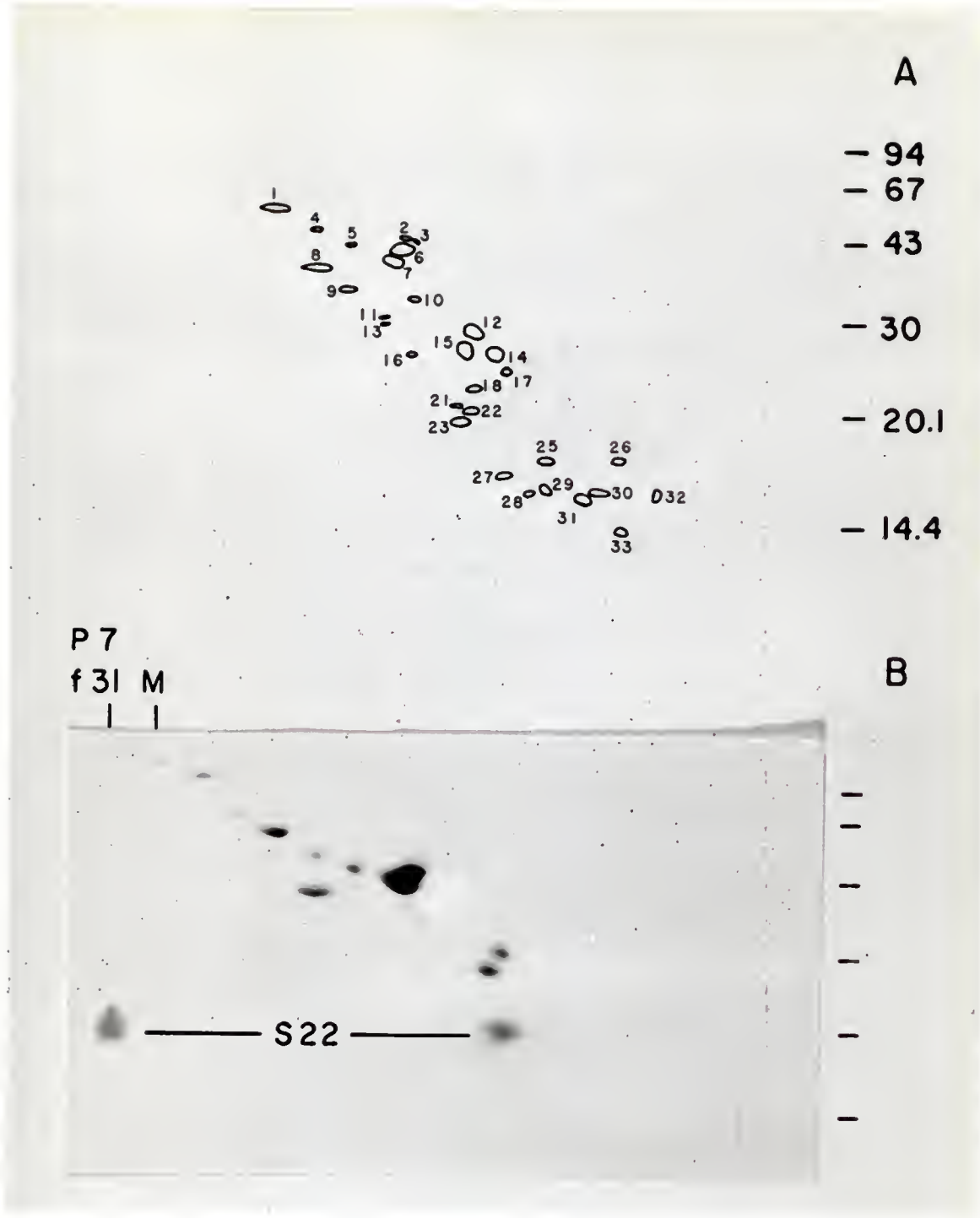


Figure 5-2 A & B Two dimensional PAGE electrophoresis of 28S ribosomal proteins coelectrophoresed with RP-HPLC fraction (f)#31 of peak (p) #7 (panel B) as shown in figure 5-1. In panel A the electrophoretic pattern for the two-dimensional separation of 28S ribosomes without any added HPLC fractions is shown to aid in the identification of ribosomal proteins for gels B to J. The same molecular weight markers (M) used in figure 3-2 were used in the following figures (A to Q).

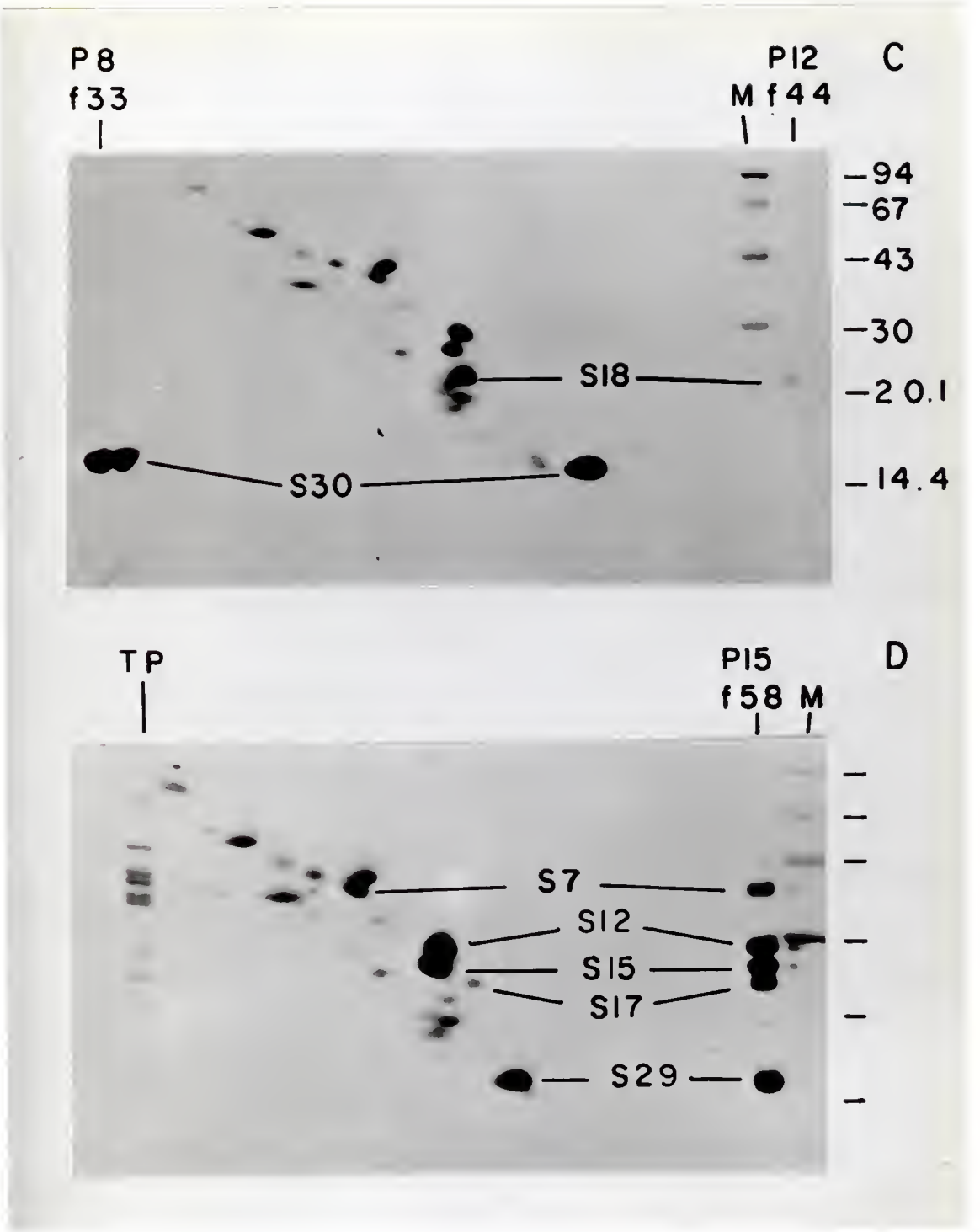


Figure 5-2 C & D Two dimensional PAGE electrophoresis of 28S ribosomal proteins coelectrophoresed with RP-HPLC fraction #33 of peak #8 and fraction #44 of peak #12 (panel C) as shown in figure 5-1. In panel D, RP-HPLC fraction #58 of peak #15 was coelectrophoresed with 28S proteins. Total protein (TP) and molecular weight markers (M) are also shown.

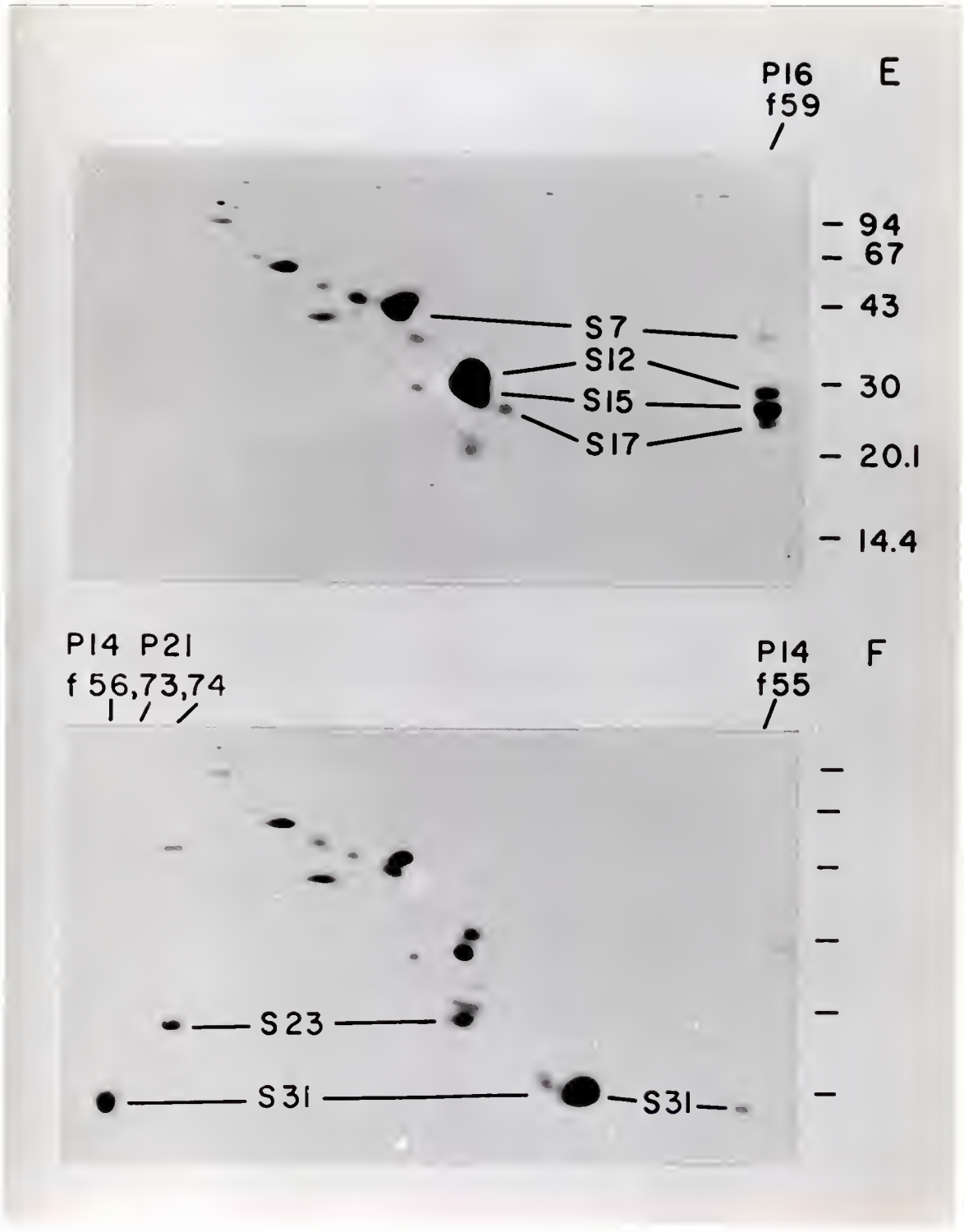


Figure 5-2 E & F Two dimensional PAGE electrophoresis of 28S ribosomal proteins coelectrophoresed with RP-HPLC fraction #19 of peak #16 (panel E) shown in figure 5-1. In panel F, RP-HPLC fractions #55 and 56 of peak #14 and fraction #73 and 74 of peak #21 were coelectrophoresed with 28S proteins.

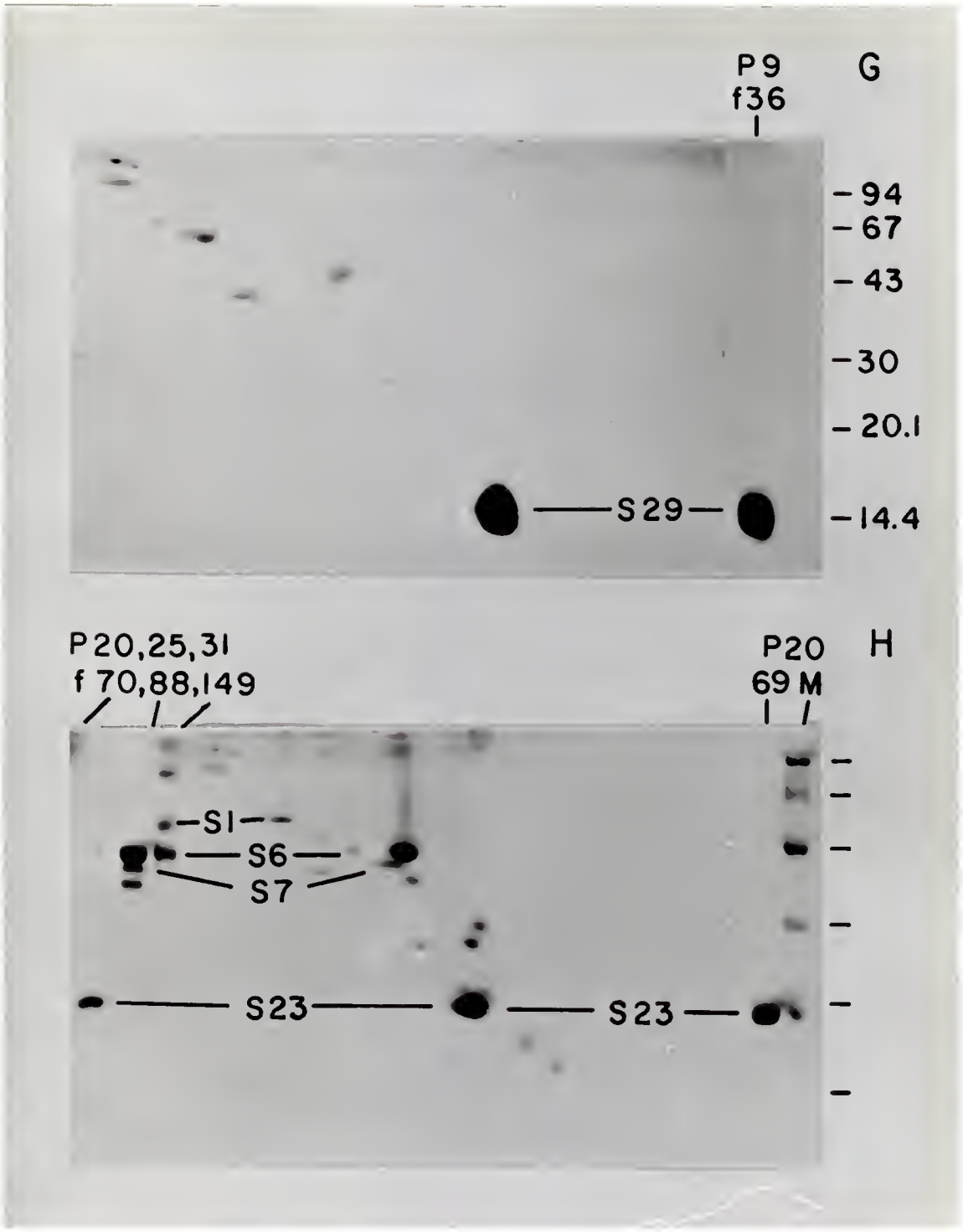


Figure 5-2 G & H Two dimensional PAGE electrophoresis of 28S ribosomal proteins coelectrophoresed with RP-HPLC fraction #36 of peak #9 (panel G) shown in figure 5-1. In panel H, RP-HPLC fractions #69 and 70 of peak #20, fraction #88 of peak #25, and fraction #149 of peak #31 were coelectrophoresed with 28S proteins.

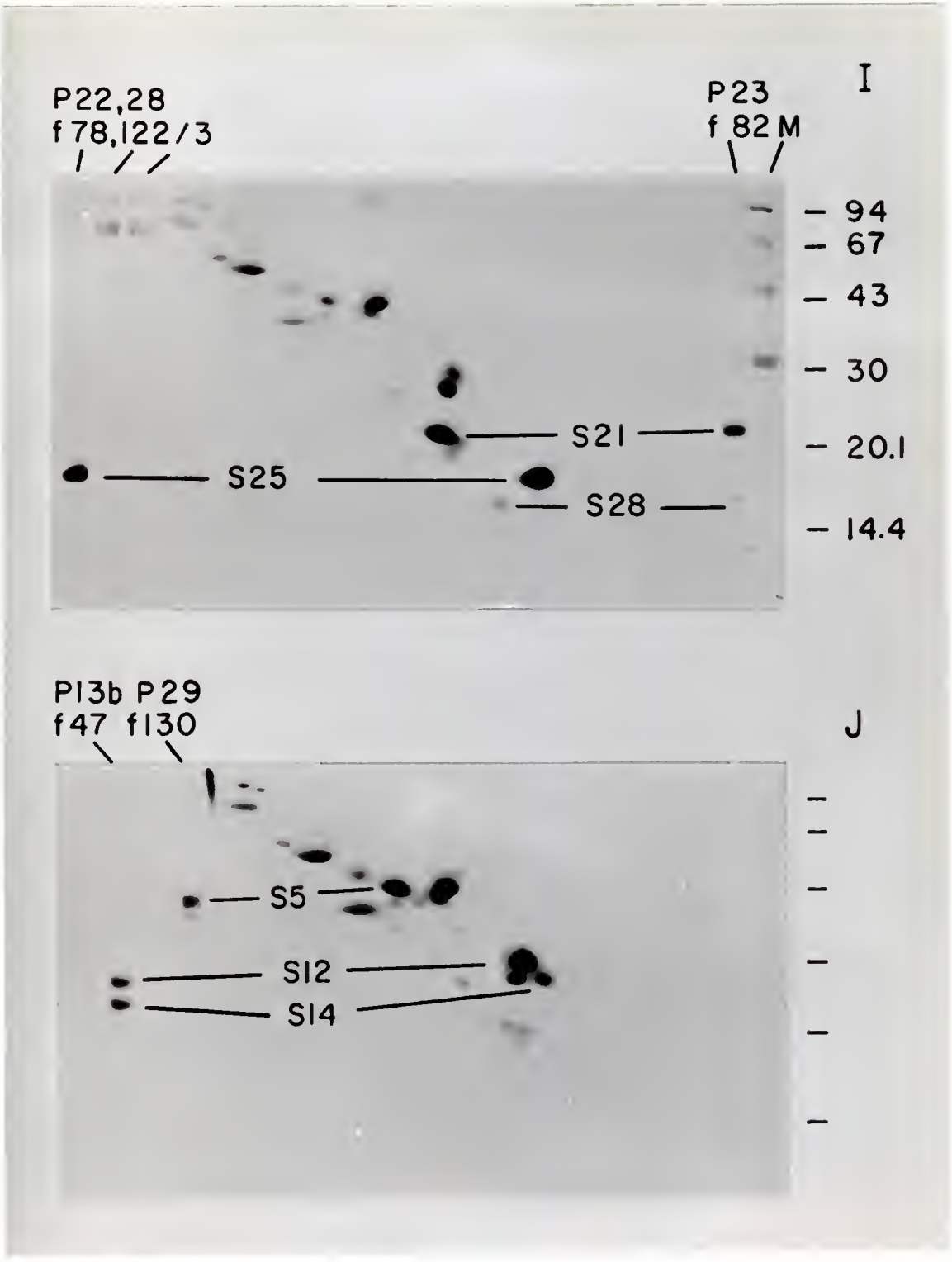


Figure 5-2 I & J Two dimensional PAGE electrophoresis of 28S ribosomal proteins coelectrophoresed with RP-HPLC fraction #78 of peak 22, fractions #122, and 123 of peak #28, and fraction #82 of peak #23 (panel I) as shown in figure 5-1. In panel J, RP-HPLC fraction #47 of peak #136, and fraction #130 of peak #29 were coelectrophoresed with 28S proteins.

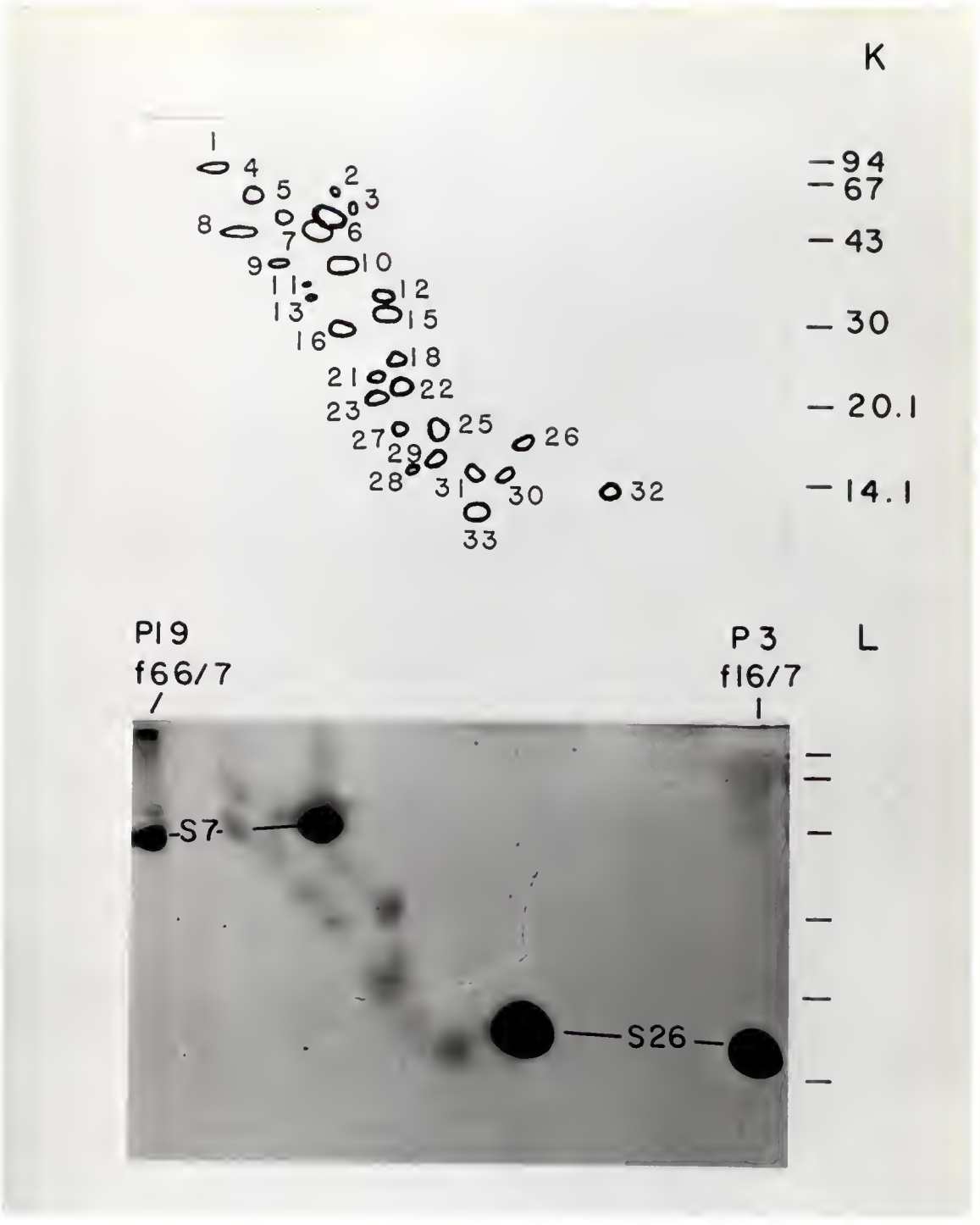


Figure 5-2 K & L Two dimensional PAGE electrophoresis of 28S ribosomal proteins coelectrophoresed with RP-HPLC fractions (f) #66, and 67 of peak (p) #19 and fractions #16 and 17 of peak #3 (panel L) as shown in figure 5-1. In panel K the electrophoretic pattern for the two-dimensional separation of 28S ribosomes without any added HPLC fractions is shown to aid in the identification of ribosomal proteins for gels L to Q.

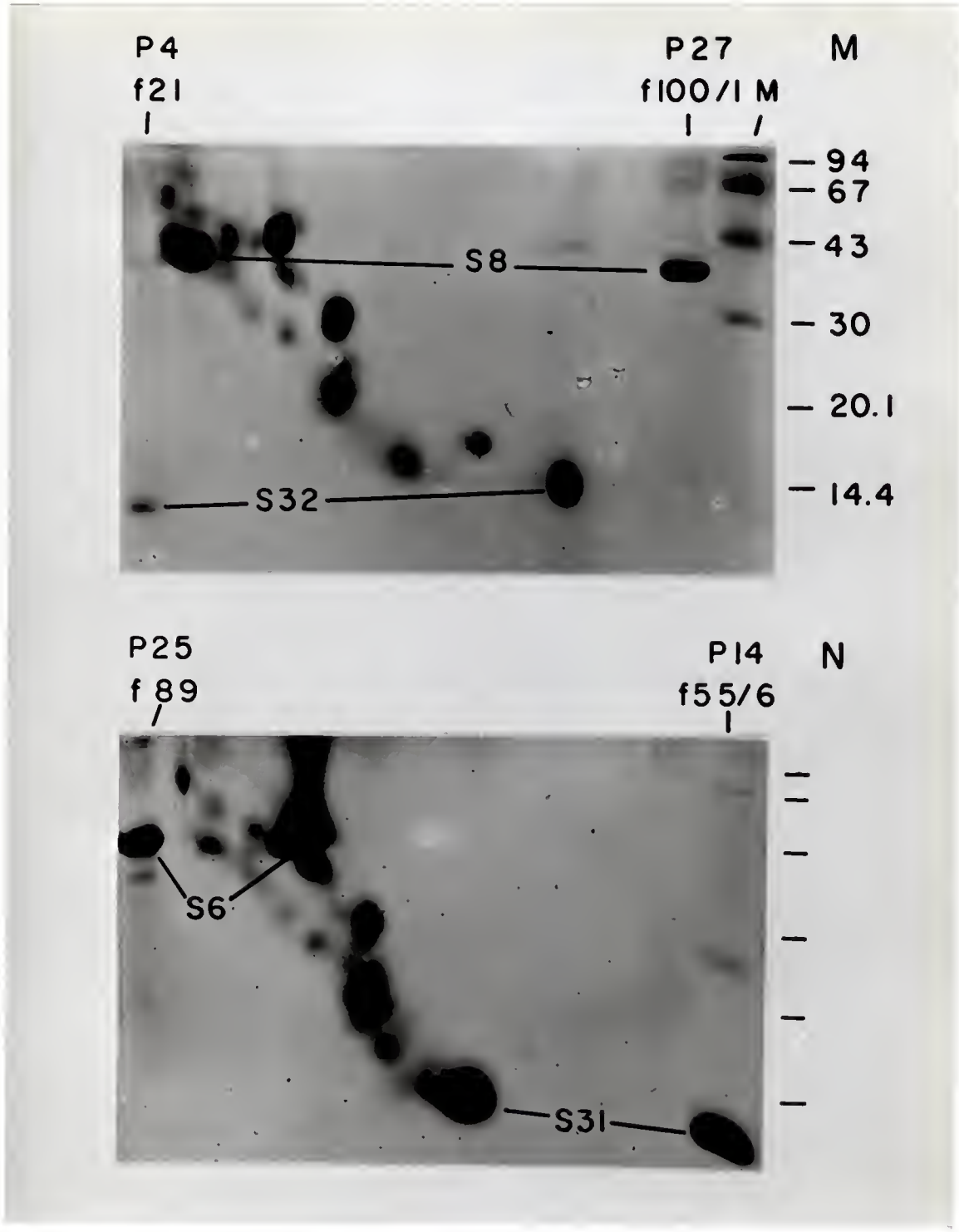


Figure 5-2 M & N Two dimensional PAGE electrophoresis of 28S ribosomal proteins coelectrophoresed with RP-HPLC fraction #21 of peak 4, and fractions #100, and 101 of peak 27 (panel M) as shown in figure 5-1. In panel N, RP-HPLC fractions #38 and 39 of peak #10 were coelectrophoresed with 28S proteins.

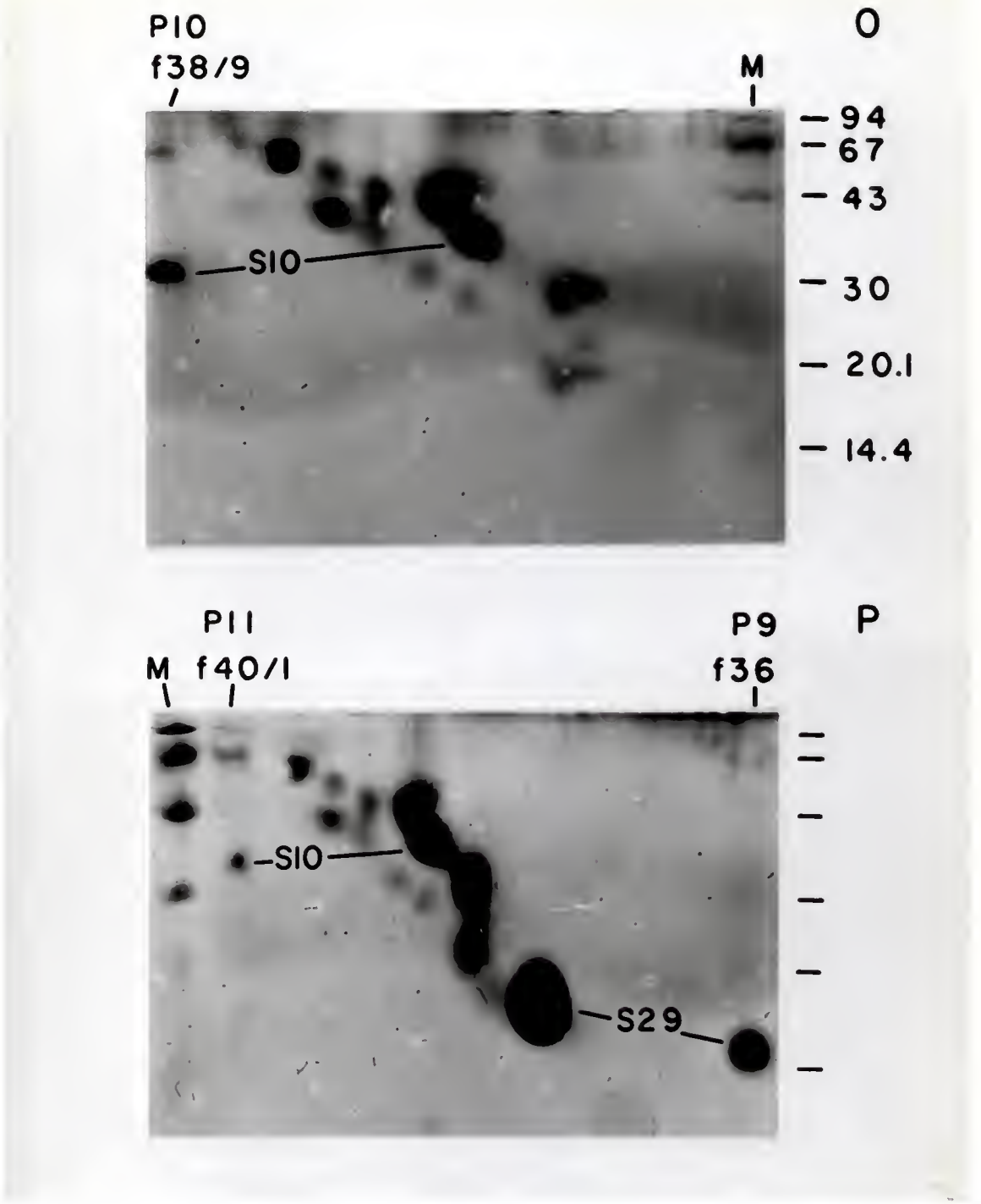


Figure 5-2 O & P Two dimensional PAGE electrophoresis of 28S ribosomal proteins coelectrophoresed with RP-HPLC fractions #40 and 41 of peak #11, and fraction #86 of peak #9 (panel O) as shown in figure 5-1. In panel P, RP-HPLC fraction #89 of peak #25 and fractions #55 and 56 of peak #14 were coelectrophoresed with 28S proteins.

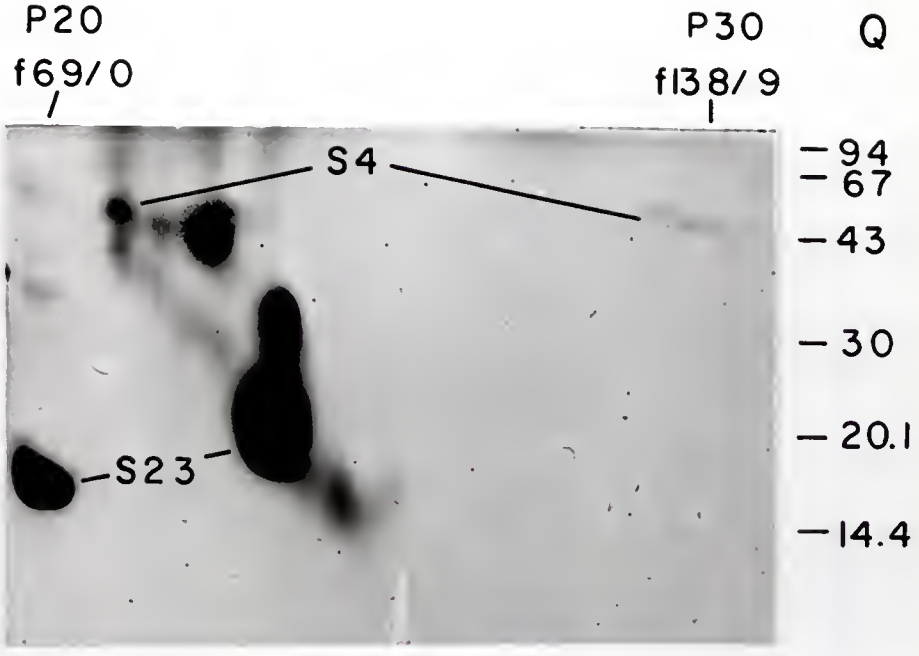


Figure 5-2 Q Two dimensional PAGE electrophoresis of 28S ribosomal proteins coelectrophoresed with RP-HPLC fraction #69 and 70 of peak #20, and fractions #138 and 139 of peak #30 (panel Q) as shown in figure 5-1.

Table 5-1

Summary of Peak Identification for RP-HPLC of 28S Ribosomal Proteins

Peak Number[a]	Fraction Number[a]	Retention Time[a] (min)	Protein Identity[b]	Pure (Y/N)[b]	2D-PAGE Gel[b]
3	16, 17	147.5 - 152.5	S26	Y	L
4	21	160.0 - 162.5	S32	Y	M
7	31	185.0 - 187.5	S22	Y	B
8	33	190.0 - 192.5	S30	Y	C
9	36	197.5 - 200.0	S29	Y	G, P
10	38, 39	202.5 - 207.5	S10	N	O
11	40, 41	207.5 - 212.5	S10	N	P
12	44	217.5 - 220.0	S18	Y	C
13b	47	220.0 - 222.5	S12, S14	N	J
14	55, 56	245.0 - 250.0	S31	Y	F, N
15	58	252.5 - 255.0	S7, S12 S15, S17 S29	N	D
16	59	255.0 - 257.5	S7, S12 S15, S17	N	E
19	66, 67	272.5 - 277.5	S7	Y	L
20	69, 70	280.0 - 285.0	S23	Y	H, Q
21	73, 74	290.0 - 295.0	S23 major S6 minor	N	F
22	78, 79	302.5 - 305.0	S25	Y	I, R
23	82	312.5 - 315.0	S21 major S28 minor	N	I
25	88, 89	327.5 - 332.5	S6 major S7 minor	N	H, N
26	91, 92	335.0 - 340.0	S16 major	N	R
27	100, 101	357.5 - 362.5	S8	Y	M
28	122, 123	412.5 - 417.5	HMr Prot[c]	N	I
29	130	432.5 - 435.0	S5	Y	J
30	138, 139	452.5 - 457.5	S4	Y	Q
31	149	480.0 - 482.5	S1	Y	H

[a]Refer to figure 5-1.

[b]Refer to figure 5-2.

[c]HMr Prot. = High molecular weight (< 70 kDa) protein that are not ribosomal proteins.

aliquots of nonimmune and anti S5 rabbit sera were diluted 1:100 with cold incubation buffer and incubated with strips of electroblotted 28S ribosomal proteins resolved on a Laemmli gel as described in Methods. In figure 5-3, a wide protein band from 37.5 to 41.5 kDa, corresponding to the Mr of S5 and possibly another protein, was immunoprecipitated by the anti S5 sera. Other minor bands were immunopre-cipitated but were no different than those seen in the nonimmune sera (figure 5-3).

To determine if the anti S5 serum recognizes S5 or possibly other 28S proteins, a 2-dimensional blot treated with S5 antiserum that had been passed through Bovine liver and E. coli lysate columns was examined. In figure 5-4, S5 was clearly the dominant protein recognized by the anti S5 serum, but to a lesser extent S6 was also recognized. Other minor proteins recognized were S15 and to a lesser extent S8, with only trace amounts of S2 and S3 being recognized. This suggests that the wide protein band noted above in the 1-dimensional immunoblot corresponds to S5 and S6. All the other bands which were also seen in the nonimmune serum correspond to other ribosomal proteins. All the minor bands that were detected in approximate equal amounts using either the nonimmune or the immune sera by 1-dimensional immunoblot, correspond well in Mr to S2, S3, S4, S8, S12, S15, and S18, which appear as minor spots on the 2-dimensional immunoblot. This observation suggests that this rabbit serum, raised

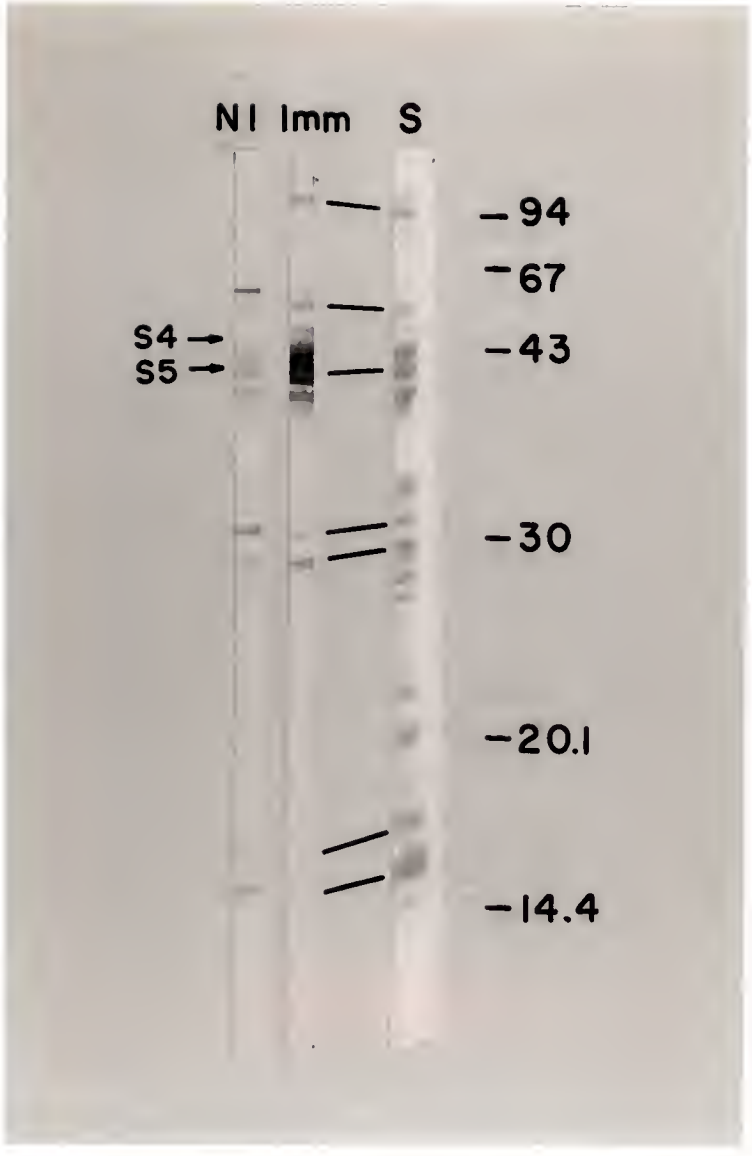


Figure 5-3 Immunoblot of 28S bovine ribosomal proteins with an anti S5 monospecific rabbit sera. 28S Ribosomes were electrophoresed on a 12% SDS-PAGE and electroblotted onto PVDF membrane. Strips of the blotted membrane containing approximately 25 pmol of ribosomal protein were incubated with non-immune (NI) and immune (Imm) rabbit sera, diluted 1:100 with low salt incubation buffer (150 mM NaCl) with 3 % bovine serum albumin at 4°C. Total 28S proteins, fast green stained (S), are shown on the right with molecular weight markers. Due to shrinkage of the PVDF membrane during fast green staining, immunoblotted and the corresponding stained proteins were marked for identification purposes. Binding of antibody to protein was visualized by a color reaction with the use of a second anti-rabbit IgG conjugated to alkaline phosphatase as described in Methods. Molecular weight markers used were the same ones described in figure 3-2. For additional experimental details see Methods.

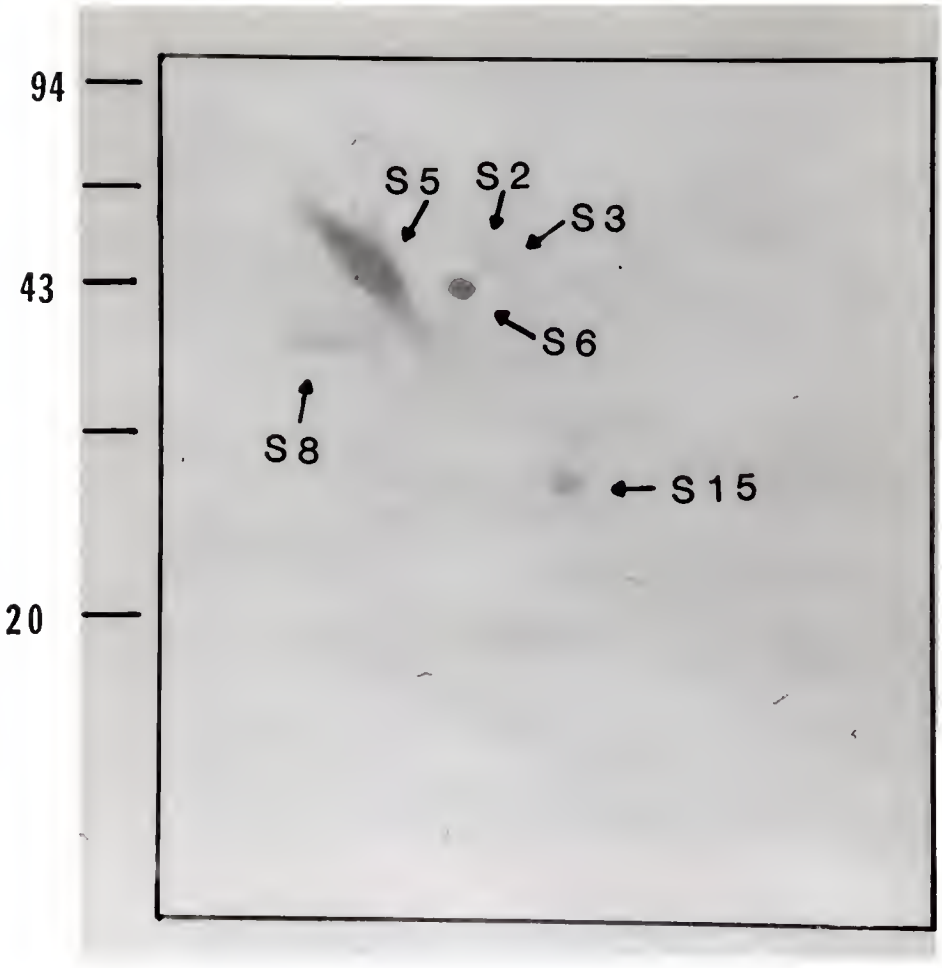


Figure 5-4 Two-dimensional immunoblot analysis of 28S bovine ribosomal proteins with an anti S5 monospecific rabbit sera. 28S Ribosomes were electrophoresed on a two dimensional PAGE and electroblotted onto PVDF membrane. Blotted membranes containing approximately 230 pmol of ribosomal protein were incubated with immune rabbit sera depleted of antibodies that bind liver and E. coli lysates. The depleted serum was diluted to a final concentration of 1:100 with low salt incubation buffer (150 mM NaCl + 3% bovine serum albumin) and incubated with the blotted membrane at 4°C overnight. Binding of antibody to protein was visualized by a color reaction with the use of a second anti-rabbit IgG conjugated to alkaline phosphatase as described in Methods. Molecular weight markers used were the same ones described in figure 3-2. S5 was the protein primarily recognized with this sera though S6 and S15 were also recognized, to a lesser degree. For additional experimental details see Methods.

against S5, will primarily recognize S5 but it is expected that other 28S ribosomal proteins, especially S6, will also be recognized to a lesser extent.

Immunoreactivity of 28S Ribosomal Proteins Resolved by RP-HPLC

Acetic acid extracted 28S ribosomes were resolved by RP-HPLC, and eluted fractions were western blotted onto PVDF membrane and probed with the anti S5 serum to verify the identity of the S5 peak. Small subunit ribosomes (658 pmol) were acetic acid extracted and resolved on a narrow bore (2.1 X 250 mm) C4 column as described in Methods. The column eluant was collected in 5 min fractions and 2.5% of each fraction was pipetted onto prewetted PVDF membrane in a 96 well dot blot apparatus. The eluant was allowed to incubate on the PVDF membrane in the dot blot apparatus for 2 h at 15°C with gentle shaking. The eluant was evacuated from the membrane under gentle vacuum for about 5 sec and allowed to dry in air for 10 min. The blot was incubated with blocking solution (3% Bovine serum) for 30 min at room temperature while shaking, and then anti-S5 rabbit serum diluted 1:100 with incubation buffer was added and shaken gently at 15° C for 10 h. Immunoreactive proteins were visualized by means of the alkaline phosphatase conjugated anti-rabbit antibody as described in Methods. Five percent of each eluted RP-HPLC fraction was also electrophoresed on a 12% polyacrylamide Laemmli gel to determine the purity of the fraction. In figure 5-5, dot immunoblots show a strong immunoreactivity of

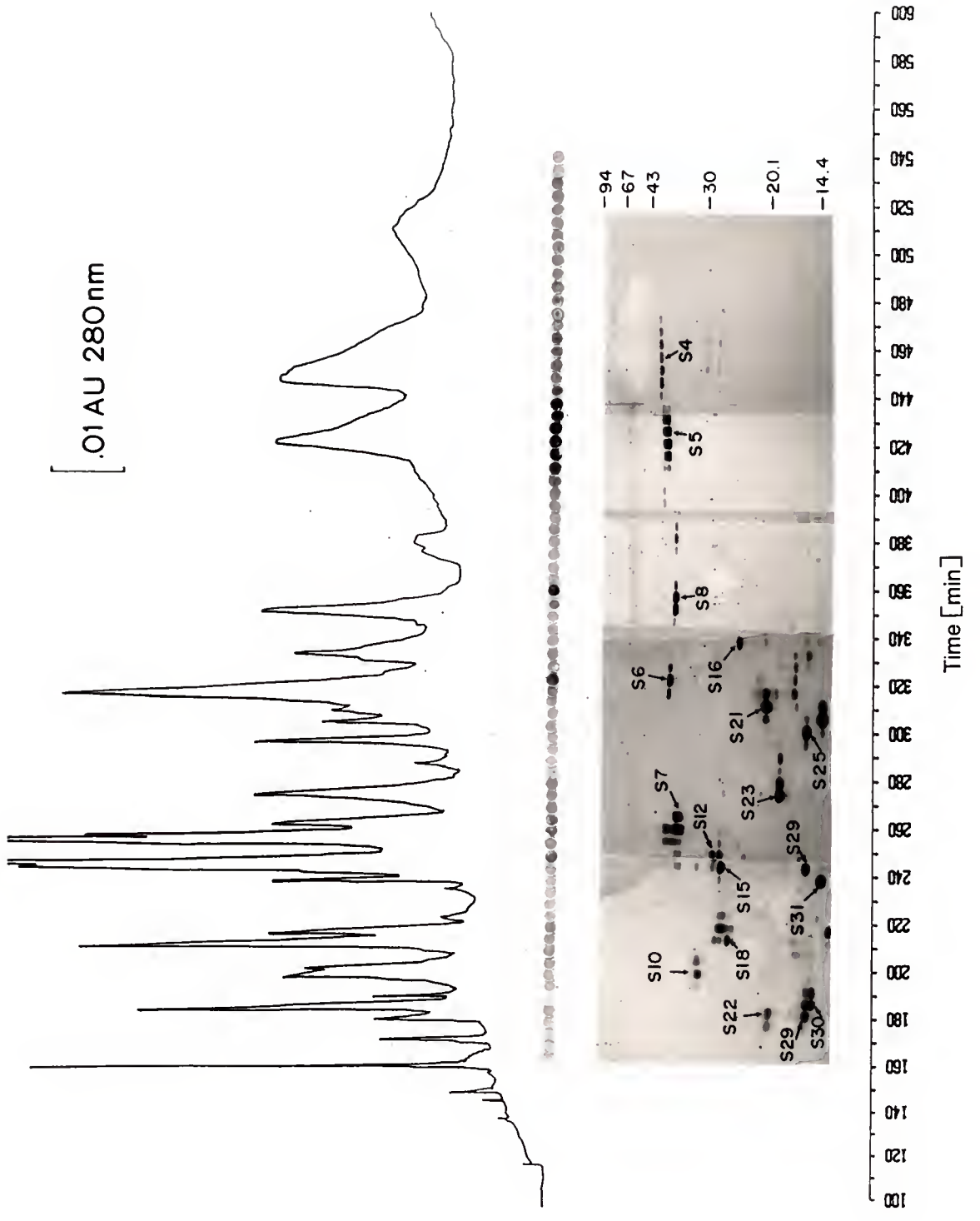
the late eluting chromatographic peak centered at 425 min. This verifies the identity of this chromatographic peak as containing S5. This peak was pure (figure 5-5, gel panel) and suitable for chemical sequence analysis. Weaker signals were seen in fractions containing S6, S8, S15, and S18, which were expected, due to the results seen in 1 and 2-dimensional immunoblots (figures 5-3 and 5-4). S5 electrophoresed as a wide band containing two closely resolved bands centered at 38 kDa.

To ensure that both bands are forms of S5, fractions from 415 to 440 min inclusive were pooled and 5% of this pooled material was analyzed by 1-dimensional immunoblot analysis (figure 5-6). This aliquot contained approximately 33 pmol of antigen and was electrophoresed on a 12% polyacrylamide Laemmli gel, then electroblotted onto PVDF membrane. Immunoblot analysis with anti-S5 serum diluted 1:100 with incubation buffer was done as described above and both closely resolved bands were strongly and equally immunoreactive (figure 5-6). This strong and equal recognition of both bands suggests strongly that both are forms of S5.

Amino Acid Sequence Analysis of S5 and Other 28S Ribosomal Proteins

Amino-terminal amino acid gas phase microsequencing of proteins was undertaken for a number of mitochondrial small subunit ribosomal proteins, including S5. All ribosomal

Figure 5-5 Immunoblot analysis of 28S bovine ribosomal proteins separated by RP-HPLC with anti S5 monospecific rabbit sera. Acetic acid extracted 28S ribosomes (658 pmol) were separated on a wide pore silica based butyl HPLC column (2.1 X 250 mm) as described in Methods and shown previously in figure 5-1. The eluant from the column was collected between 160 to 560 min at 5 min intervals. Approximately 1% of the eluant (6.6 pmol) from each fraction was blotted onto PVDF membrane with the use of a 96 well dot blot apparatus. The blotted membrane was incubated with anti-S5 monospecific rabbit sera, diluted 1:100 with low salt incubation buffer (150 mM NaCl) containing 3 % bovine serum albumin at 4°C overnight. Binding of antibody to protein was visualized by a color reaction with the use of a second anti-rabbit IgG conjugated to alkaline phosphatase as described in Methods. Approximately 10% of each fraction from the HPLC eluant was analyzed by 12% SDS-PAGE as shown at the bottom of the figure. The identity of many of the proteins is also indicated, when known. Molecular weight markers used are the same ones described in figure 3-2.


.01 AU 280nm
-94
-67
-43
-30
-20.1
-14.4
S4
S5
S8
S6
S16
S21
S25
S7
S12
S23
S29
S15
S31
S10
S18
S22
S29
S30
100
120
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540
560
580
600
Time [min]

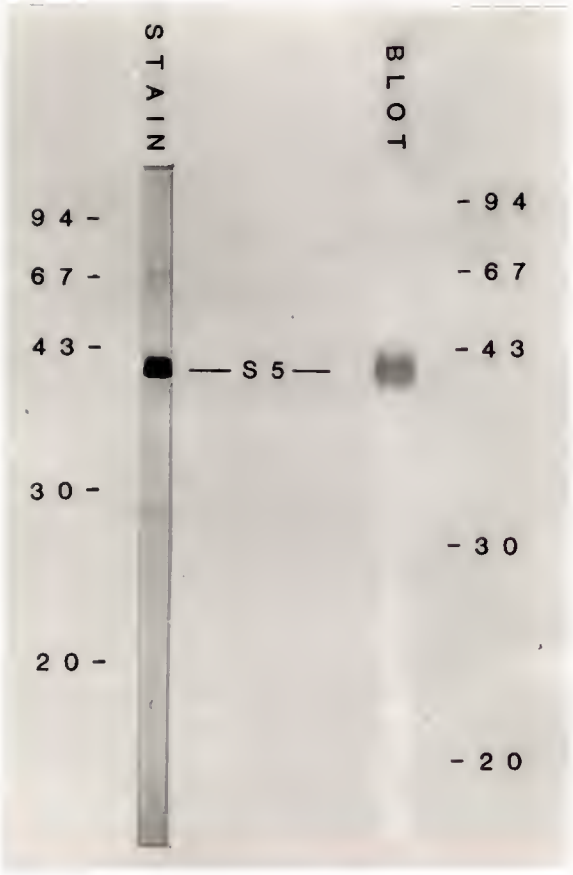

Figure 5-6 Immunoblot analysis of S5 protein isolated by RP-HPLC. Fractions eluted from 415 to 440 min inclusive, were pooled and approximately 5% was electrophoresed by 12% SDS-PAGE and electroblotted onto PVDF as described in Methods. The blotted membrane was incubated with anti-S5 monospecific rabbit sera, diluted 1:100 with low salt incubation buffer (150 mM NaCl) with 3 % bovine serum albumin at 4°C overnight. Binding of antibody to protein was visualized by a color reaction with the use of a second anti-rabbit IgG conjugated to alkaline phosphatase as described in Methods. The immunoblotted protein (BLOT) is compared to a silver stained (STAIN) lane to indicate the purity of the protein fraction. The chromatographic peak contained greater than 98% pure S5 protein which was recognized by the anti-S5 monospecific antibody. The remainder of this pooled sample was used to obtain amino acid sequence by Edman degradation chemistry.

proteins were sequenced on PVDF membrane supports and table 5-2 summarizes the methods employed. Proteins isolated in pure form from the RP-HPLC were deposited onto discs (1 cm diameter) of PVDF membrane by either being spotted onto or bathing discs in the eluant. If pure fractions were not available, the protein or peptides were electrophoresed and electroblotted onto PVDF for sequencing as described in Methods.

For one protein, S5, amino terminal sequencing failed to produce any sequence even though two attempts were made under conditions where other proteins were successfully sequenced (Table 5-2). Chemical cleavage was then done to obtain internal sequence information and was accomplished by chemical digestion at methionyl residues with cyanogen bromide. This generated 6 peptides (figure 5-7), in addition to the full length S5 protein. All six CNBR peptides were sequenced successfully. Several were redundant sequences, suggesting partial cleavages with the chemical agent, but three unique sequences were found (table 5-3). One disadvantage to sequencing proteins blotted onto PVDF supports is a reported loss of tryptophane residues during electrophoresis or blotting (Matsudaira, 1987). Sequence analysis of the 13 kDa CNBR peptide of S5 revealed that at residue 5 no appreciable amount of PTH-AA could be detected above background levels (figure 5-8 A). There was apparently PTH-Trp, but the amount was not significantly above background levels. Also a new

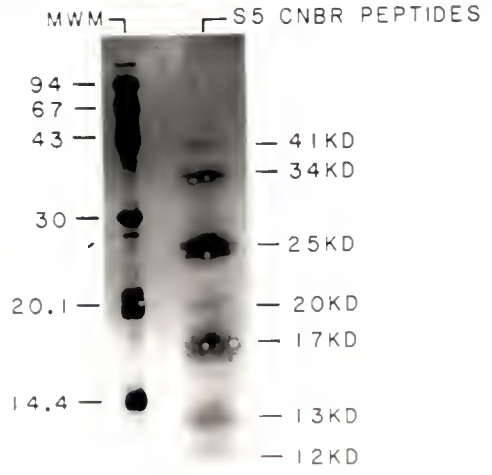

Figure 5-7 Electrophoretic separation of cyanogen bromide peptides of S5. The peptides were generated in the presence of 70% formic acid and CNBR as described in Methods. The resulting peptides were resolved by 15% SDS-PAGE and electroblotted onto PVDF membrane. The peptides were visualized by coomassie staining and the peptide bands excised and analyzed by gas phase chemical amino acid sequencing as shown in table 5-3. The molecular weight markers are the same as shown in figure 3-2.

TABLE 5-2

Summary of Ribosomal Proteins and Peptides Submitted for Gas Phase Amino Acid Sequence Analysis

Protein or Peptide	Preparation for Sequencing[a]	Number of residues Sequenced	Repetitive Yield (%)	Amount initial-AA (pmol)
S4	Spotted onto PVDF	30	96.1	Leu 136
S8	Bathed in eluant	20	92.9	Trp? 5
S25	Spotted onto PVDF	34	97.2	Lys 200
S5	Electroblotted[b]	0	-	-
S5	Electroblotted[c]	0	-	-
CNBR Peptides of S5:				
25 kDa	Electroblotted	36	94.1	Val 11
12 kDa	Electroblotted	37	89.4	Arg 16
13 kDa	Electroblotted	37	90.5	Val 31

[a]See Methods for experimental details. S5 and CNBR peptides of S5 were resolved on 12 and 15% Laemmli polyacrylamide gel, respectively and electroblotted onto PVDF membranes as described in Methods.

[b]Following 5 sequencing cycles the protein content remaining on the PVDF membrane was hydrolysed in HCl and analyzed by amino acid analysis and 17 pmol of S5 remained on the membrane.

[c]Following electroblotting of S5 onto PVDF membrane, one third of the membrane containing the blotted S5 protein was cut off and analyzed for protein content by AA analysis. Based upon the amino acid analysis 20 pmol of protein was on the membrane submitted for sequence analysis.

peak was detected eluting just prior to dithiothreitol (DTT) in the chromatogram (figure 5-8 A). It was suspected that this peak was an oxidized product of Tryptophane, as reported by Walsh et al. (1988), but the peak we observed eluted much later, and due to differences in chromatography methods we were not able to make an assignment for residue 5. Since a

TABLE 5-3

Summary of Amino Acid Sequence for Several Bovine Mitochondrial Ribosomal Proteins and CNBR Peptides of S5

Protein	Amino Acid Sequence
S4	H_2N-L-L-S-A-A-Y-E-D-S-R-K-W-E-A-R-A-K-E-D-S-H-L-A-D-A-A-X-X-M-H-
S8	H_2N-W?-S-E-A-E-S-G-S-P-K-I-K-K-P-T?-F-M-D?-E-E-V?-
S25	H_2N-K-N-R-A-A-R-V-R-V-G-K-G-N-K-P-V-T-Y-E-E-A-H-A-P-P?-Y-I-A-H?-R-K-G-G?-L-
CNBR Peptides of S5:	
25 kDa	-V-R-K-P-A-L-E-L-L-H-Y-L-K-N-T-N-F-A-H-P-A-V-R-Y-V-L-Y-G-E-K-G-T-G-K-T-L-
34 kDa and 17 kDa CNBR peptides have the same sequence as the 25 kDa peptide.	
12 kDa	-R-V-R-N-A-T-D-A-V-G-I-V-L-K-E-L-K-R-Q-S-S-L-G-V-F-R-L-L-V-A-V-D-G-V-N-A-L-
20 kDa has the same sequence as the 12 kDa peptide.	
13 kDa	-V-K-N-D-W-Q-G-G-A-I-V-L-T-V-S-Q-T-G-S-L-F-K-P-R-K-A-Y-L-P-Q-E-L-L-G-K-E-G-

[a]One letter code for amino acids used in this figure

number of oxidized products of Tryptophan exist, an empirical approach was used in an attempt to determine if residue 5 was indeed PTH-Tryptophane.

A test protein of known amino acid sequence (containing Trp) was subjected to CNBR digestion and blotting onto PVDF in a manner identical to that used for CNBR peptides of S5. This was done to determine if Trp residues are lost and if so were

oxidized products of Trp present to indicate the presence of Trp. Sperm whale myoglobin was used as a test protein because its amino acid sequence was known and it has a Trp residue near its amino-terminus (residue #7), to reduce the number of cycles required to sequence before encountering a PTH-Trp residue. In one treatment, 600 pmol of myoglobin was digested by CNBR and the second sample of the same amount of myoglobin, was not digested. Both samples were blotted onto PVDF in the same manner as described for S5 and submitted for AA-sequence analysis. Sequence analysis of residue 7 from undigested myoglobin revealed 2.5 pmol of PTH-Trp and no new peak (figure 5-8 C) observed just prior to DTT as observed in residue 5 of the CNBR peptide of S5 (Figure 5-7). This amount of PTH-Trp was about half of the amount of other PTH-AA found in that analysis. The large loss of material was due to losses in transfer of protein to PVDF. However AA-sequence analysis of this same residue following CNBR digestion revealed that PTH-Trp was not found in amounts above background and the putative oxidized PTH-Trp peak appeared at the same retention time, just prior to the DTT peak (figure 5-8 B), as observed in the sequencing of the 13 kDa CNBR peptide of S5. This suggests strongly that Trp residues do not survive CNBR cleavage, but under the conditions used in this study do survive electrophoresis and electroblotting. Though these results do not agree with the observations made by Matsudaira (1987), the full extent of differences in electrophoretic and blotting methods used could not be determined from the publication.

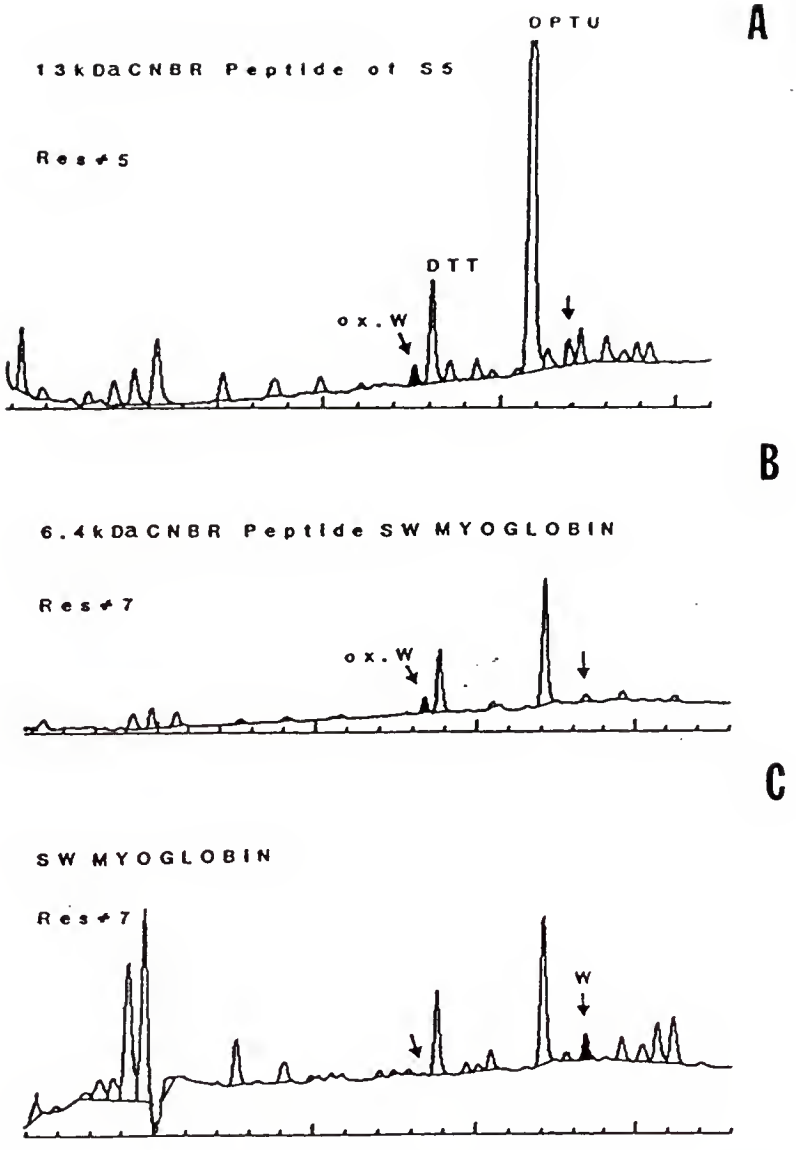

Figure 5-8 Elucidation of a putative oxidized tryptophan residue found in the 5th PTH amino acid residue of the 13 kDa CNBR peptide of S5. This was done by comparing the PTH tryptophan residue #7 of sperm whale myoglobin which was chemically digested by CNBR (panel B) in an identical manner as S5 (panel A) and with no chemical digestion (panel C). All three panels are HPLC chromatograms of PTH amino acid residues resulting from Edman degradation chemistry of the respective peptides and proteins. In all cases PTH analysis of residues was done using an IBI 470 gas phase amino acid sequencer with a model 120 A on-line PTH-HPLC analyzer. The arrows indicate the presence of either oxidized tryptophan (panels A & B) or tryptophan PTH residues (panel C). For additional experimental details see Methods.

One likely difference that may account for the observation was that in the electrophoresis of Laemmli gels, 0.1 mM thioglycolate was added to the cathode run buffer in our method and not in the method reported by Matsudaira (1987). Thioglycolate functions as a free radical scavenger to reduce the possibility of N-terminal blockage and chemical modifications to other residues and presumably protected Trp residues during electrophoresis and blotting procedures.

The presence of the oxidized PTH-Trp residue, just prior to the DTT peak, in CNBR peptides of Sperm Whale myoglobin and 13 kDa peptide of S5 strongly suggest that residue 5 in the peptide of S5 is PTH-Trp.

Discussion

I have identified the GTP-binding protein in bovine mitochondrial ribosomes. This finding is without precedent in other ribosome systems. Procedures and strategies were developed for the purification and microsequencing of S5 in order to gain insight into the possible functional role of this protein on the small subunit of mitochondrial ribosomes. Additionally the primary sequence of mammalian mitochondrial ribosomal proteins, which have never been reported in the literature, are presented here. These sequences are partial amino-terminal sequences of several mitochondrial ribosomal proteins, as well as primary sequences from CNBR generated peptides of S5 (Table 5-3).

Approximately one-third of the primary sequence of S5 has been determined and two limited sequences discovered (GX_4GKT and DAVGIVL) probably contribute to the GTP binding domain in S5, by virtue of their homology to corresponding sequences in other GTP-binding proteins. These two sequences are homologous to sequences found in the A and E sites which bind to the phosphate side chain and the guanine base, respectively (Figure 5-9). The sequence (GX_4GKT) found in the 25 kDa CNBR peptide of S5 is identical to that same limited sequence found (A site) to interact with the α and β phosphates of GTP in all GTP-binding proteins (table 5-4).

Residues in the E site are not as highly conserved as those in the A site in all GTP binding proteins; nonetheless, if conserved substitutions (Dayhoff, 1978) are allowed, this limited sequence found in S5 (found in the amino terminus of the 12 and 20 kDa CNBR peptides, Table 5-3) is homologous to the corresponding sequence found in the E site of other GTP-binding proteins as shown in Table 5-4. Though this comparison was made to those GTP-binding proteins that interact with ribosomes and to a limited number of residues, they were to sequences that have been found to be highly conserved and demonstrated to be functionally important in their interactions with GTP. A computer search using the entire known sequence for S5 CNBR peptides and other ribosomal proteins (S8, S4, and S25) showed no significant homologies with any other proteins in the GENEBANK data bank.

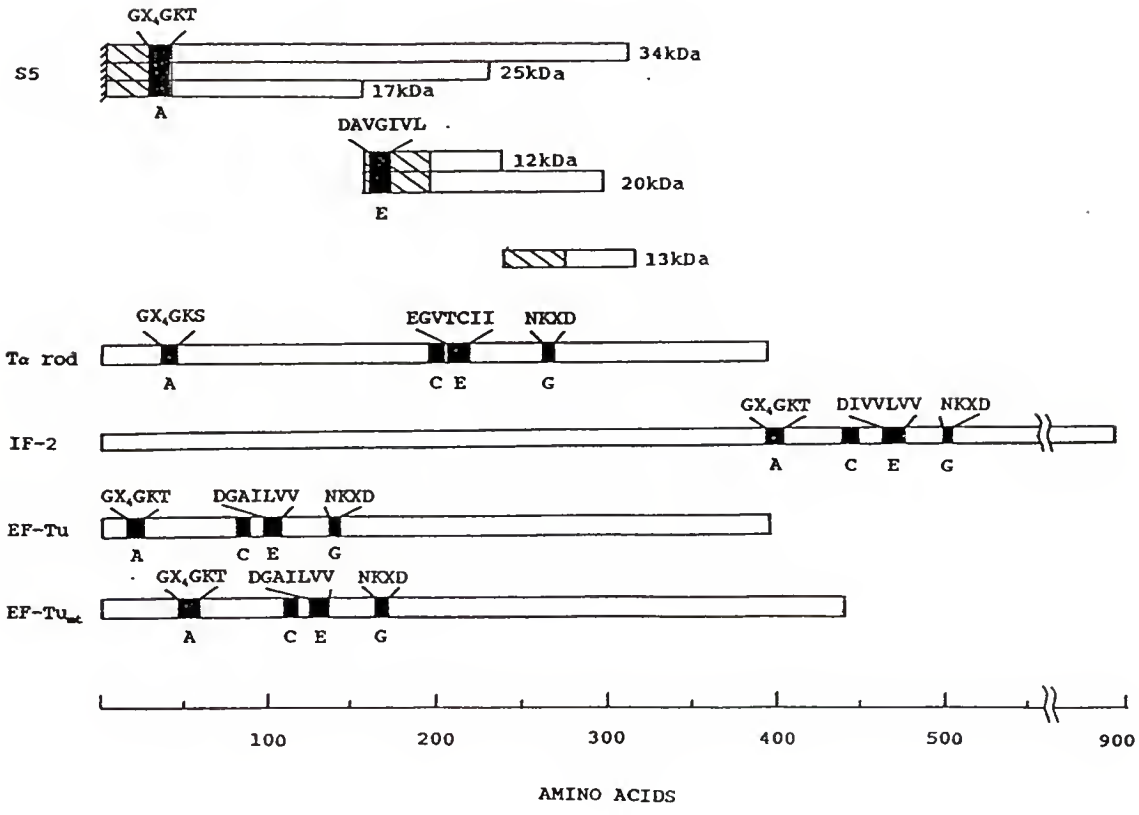

Figure 5-9 Comparison of putative conserved regions found in the amino acid sequence of CNBR peptides of S5 to homologous regions found in other GTP-binding proteins. The putative A site and E site which have been shown to interact with the pyrophosphate and guanine base of GDP respectively in other GTP-binding proteins are shown for S5 and the corresponding regions in other known GTP-binding proteins. For references see figure 1-1. A putative peptide map for S5 is shown based on overlapping sequence found among the CNBR peptides their size and the total molecular weight of S5. Portions of the CNBR peptides that have been sequenced by chemical means are shown as hash marked areas.

Table 5-4

Comparison of Amino Acid Sequence found in Mitoribosomal Protein S5 with those corresponding to the E site found in other GTP-binding proteins involved in Protein Synthesis

Protein[a]	E Site[b]	Homology[c]
S5	D-A-V-G-I-V-L	
IF2	D-I-V-V-L-V-V	71%
EF-Tu	D-G-A-I-L-V-V	71%
EF-G	D-G-A-V-M-V-Y	57%
$EF\text{-}Tu_{mt}$	D-G-A-I-L-V-V	71%

[a] The source of these proteins are: S5, Bovine mitochondrial; IF2, EF-Tu, and EF-G, E. coli; $EF\text{-}Tu_{mt}$ S. cerevisiae mitochondrial. See figure 1-1 for references.

[b] Conserved substitutions (Dayhoff, 1978) were allowed in making comparisons. Underlined residues (one letter code) are those differing from the sequence in S5. For a more exhaustive comparison refer to figure 1-1 (E site). Dayhoff (1978) conservative categories are as follows: C; S,T,P,A,G; N,D,E,Q; H,R,K; M,I,L,V; F,Y,W.

[c] The percent homology of amino acid sequence of S5 are compared to the corresponding region in other GTP-binding proteins shown above. Conserved substitutions were allowed as described in [b].

CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

Mammalian mitochondrial ribosomes are known to differ from other procaryotic, eucaryotic cytoplasmic, and even other mitochondrial ribosomal systems in translocase specificity and recognition of mRNA. O'Brien et al. (1990) discovered yet another unusual characteristic of this ribosome, binding of GTP directly to an integral site on the mitochondrial ribosome. In this study I have further characterized this high affinity binding and found that it is specific for GTP and GDP. We now have an understanding of the minimal molecular characteristics required for high affinity binding by comparing binding affinities of GTP for the 28S ribosome to other closely related nucleotides such as ITP, GMP, and 8-Azido GTP, which bind to the ribosome with reduced affinity, and other nucleotides (ATP, CTP, UTP, and periodate cleaved GTP) which do not bind at all. This high affinity binding could tolerate high salt concentrations, implying the binding is not electrostatic in nature and persists at salt concentrations that strips the ribosome of putative translocases and even to the point where the integrity of the ribosome begins

to be lost (O'Brien, et al., 1990) suggesting strongly that GTP binds directly to the 28S ribosome.

Probing deeper into this binding phenomenon, we identified the protein that binds GTP on the ribosome to be S5. This was accomplished by photochemically labeling the GTP binding site on the ribosome with a photoreactive analog of GTP and GTP itself. Both ligands specifically radiolabeled S5 following photoactivation by uv irradiation of the bound ligand on the 28S ribosome. Ligand specificity for the photoaffinity labeling of this ribosome was consistent with the results of filter binding assays, strongly suggesting that S5 specifically bound only GTP and GDP.

Micro methods were developed to purify, identify and chemically sequence not only S5, but other mitochondrial ribosomal proteins as well. Though none of these proteins were shown to have any significant overall homologies to other ribosomal proteins in other systems, strong homologies were found to two sites of 7 to 8 residues in S5 to conserved areas found in all GTP-binding proteins. The first of these sequences found in S5, GX_4GKT, is known to interact with the pyrophosphate group of GDP and involved in GTPase activity in other GTP-binding proteins. The other sequence, DAVGIVL, was found to be homologous to sequences that interact with the guanine base in GTP-binding proteins. Though only a third of the S5 protein has been sequenced so far the identification of at least two of the four highly conserved regions found in

GTP-binding proteins suggests that GTP binding in S5 may be similar in its molecular detail as in other GTP-binding proteins.

Efforts to find and clone the gene for S5 are in progress. A number of approaches to obtaining this gene are being pursued. Screening of cDNA expression libraries from bovine liver and kidney and human hepatoma cells with the anti S5 monospecific antibody, and probe screening a cDNA library with redundant oligonucleotides complementary to the known amino acid sequence of S5. An attempt is being made to amplifying the S5 gene, using redundant oligonucleotide primers also complementary to the known amino acid sequence of S5, by the polymerase chain reaction from the above cDNA or genomic libraries. This would provide not only the first complete sequence of a mammalian mitochondrial ribosomal gene to compare to other ribosomal proteins, but allow for a complete comparison of S5 to other GTP-binding proteins.

The function of this GTP/GDP binding activity is unknown. However, the fact that it binds to the small subunit leads one to speculate that it is involved in protein synthesis initiation complex formation, or possibly acting as a guanine nucleotide exchange factor.

One possibility is that S5 is a resident IF2 like molecule. In support of this hypothesis, it is known that procaryotic IF2 forms a ternary complex with GTP, and aminoacyl tRNA, on the 30S ribosome. The order of binding for

mRNA, tRNA, IF2, and 30S ribosome to form a competent initiation complex is unimportant (Guarlerzi, 1986). In contrast, in eucaryotic cytoplasmic ribosomes the binding of the above components to eIF2 is tightly controlled (Dholakia and Wahba, 1989) and a guanine exchange factor (GEF) mediates transfer of the GTP/GDP molecule to the eIF2 prior to binding the 40S ribosome to form the initiation complex. In addition to GEF a number of accessory proteins exist to control initiation complex formation and ultimately protein synthesis. In mitochondrial ribosomal systems this degree of control over protein synthesis may be even more relaxed. This would also coincide with the fact that mitochondrial ribosome contain nearly twice the number of proteins as procaryotic ribosomes implying that at least some of these additional proteins may have functional roles in protein synthesis. Studies are underway to determine if mRNA and or mt-initiator tRNA interact with S5 on the ribosome which would help substantiate the above hypothesis.

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BIOGRAPHICAL SKETCH

John Claude Anders was born April 21, 1951, in Miami, Florida. He earned his Bachelor of Science degree in December of 1974, majoring in biological sciences at Florida State University. He also was married to Valerie M. McGee in August of the same year. In December of 1978 he earned a Master of Science in toxicology from Auburn University and also was commissioned as a second lieutenant in the Alabama Army National Guard in July of that same year. He accepted an active duty commission as a first lieutenant in the Medical Service Corp of the U.S.Army in November of 1978 and became a member of the Department of Pharmacology in the Division of Experimental Therapeutics at the Walter Reed Army Institute of Research. There he studied the biotransformation and toxicity of various experimental antimalarial and antileishmanial drugs being developed for human use. In June of 1985, he continued his graduate education in the Department of Biochemistry and Molecular Biology at the University of Florida, Gainesville, working under the direction of Dr. Thomas W. O'Brien. Presently Major Anders is a principal investigator in the Department of Immunology at the Walter Reed Army Institute of Research, Washington D.C. He is working with a group that is attempting to develop a malarial vaccine for use in humans.

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